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13. ABSTRACT (Maximum 200 Words)
The aim of this project is to determine which parts of the matrix metalloproteinase (MMP) genes cause those genes to be over-expressed in breast cancer, contributing to invasion and metastasis. It was determined that breast cancer cells can be classified into two types: one type retains its epithelial characteristics, the other has lost them by undergoing an epithelial-mesenchymal transition (EMT). The MMPs in each cell type are up-regulated by distinct molecular mechanisms. Gelatinase B is produced at an unusually high level by the epithelial cells, requiring no stimulus from the other cell type. In contrast, the cells that have undergone an EMT up-regulate their MMPs in response to a factor secreted by the epithelial cells. Using MMP promoters linked to measurable reporter genes, it was determined that the constitutively high levels of gelatinase B production are mediated through the region upstream of the proximal promoter, whereas the high inducible levels of MMP production in the cells that have undergone an EMT are not mediated through the upstream regions. Thus, the mechanism determining high MMP production by breast cancer cells depends on the state of differentiation of the cells.

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FOREWORD

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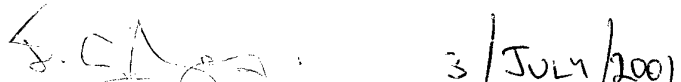

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(5) INTRODUCTION

The critical feature of breast cancer that makes it a malignant disease is its ability to spread from the breast to other parts of the body. It is these secondary breast cancer tumors whose growth and destruction of surrounding tissues can cause suffering and the death of the patient. The spread of the tumor, in the context of both invasion of tissues locally and metastasis to distant organs, requires the destruction of the extracellular matrix (ECM). The purpose of this project is to identify the molecular and genetic basis for this abnormal ECM destruction by addressing the expression of the genes of a group of proteinases, the matrix metalloproteinases (MMPs), which are made at high levels in and around carcinomas (Chambers and Matrisian, 1997; Stetler-Stevenson et al., 1993; Westermarck and Kähäri, 1999). Specifically, the gene control regions of the MMP genes that are responsible for their high level expression in carcinoma cells will be identified. This is to be achieved by transfecting carcinoma cells with plasmids that contain reporter genes driven by wild-type or mutant MMP promoters to localize the control elements to specific regions of the MMP genes and verifying their roles *in vivo*.

(6) BODY

Results

Quantitative PCR assays for measuring expression of reporter genes in cell and tissue samples.

Quantitative PCR assays have been developed for the measurement of absolute numbers of mRNAs for MMPs and tissue inhibitors of metalloproteinases (TIMPs) that, for the first time, permit the determination of absolute numbers of specific mRNA molecules within cell and tissue samples (Martorana et al., 1999)(Appendix 1). Quantitative PCR assays were also developed for mRNAs derived from the neo and chloramphenicol acetyl transferase genes used as reporters for wild-type and mutated promoters, respectively. These are more sensitive than the activity-based assays commonly employed for these reporter genes and less likely to suffer from interference by other proteins in the sample, such as deacetylases.

Epithelial cells up-regulate matrix metalloproteinases in cells within the same mammary carcinoma that have undergone an epithelial-mesenchymal transition (EMT).

The BC1 cell line that is being used as a model for breast cancer is composed of epithelial cells that have retained their epithelial morphology and metaplastic cells that have not. We used the novel quantitative PCR method to determine absolute numbers of mRNA molecules of MMPs and TIMPs in cell and tumor tissue samples for the first time. This enabled us to identify the source and degree of mRNA induction in BC1 when these 2 cell types are co-cultured. Collagenase-3, stromelysin-1 and stromelysin-2 were

predominantly produced by metaplastic cells, whereas gelatinase B, TIMP-1 and TIMP-2 were produced by both cell types. Collagenase-3, stromelysin-1, gelatinase B and TIMP-2 were present in cell culture and tumor tissue samples at a level of >50 copies per cell, indicating that they are major gene products, because <2% of genes are expressed at this level. The mRNA levels of collagenase-3, stromelysin-1, stromelysin-2 and gelatinase B were all substantially up-regulated in the metaplastic cells by co-culturing them with the epithelial cells (Table 1)(Martorana et al., 1998)(Appendix 2).

The two cellular phenotypes have been characterized in more detail by analysis of their protein and mRNA expression profiles, to determine how they correspond to phenotypes within human neoplasms. Key differences between the two cell types are that the epithelial cells are positive for E-cadherin, keratin, β4-integrin subunit, the keratinocyte growth factor-binding splice isoform of fibroblast growth factor receptor-2 (FGFR2), whereas the metaplastic cells express the basic FGF-binding splice isoform of FGFR2 (Figure 1), are negative for the other aforementioned mRNAs and have up-regulated vimentin protein levels. These data suggest that the metaplastic cells have undergone an EMT. Preliminary experiments are underway to induce the epithelial cells to undergo an EMT *in vitro*, so that the relationship between this phenomenon and the induction of MMP genes via modulation of their promoters can be studied in more detail.

Table 1. Summary of MMP and TIMP gene expression by BC1 E-cells and M-cells grown either alone or in co-culture.

MMP/TIMP	E-cells alone	E-cells co-cultured	M-cells alone	M-cells co-cultured
Stromelysin-1	-	-	-	+++
Stromelysin-2	-	-	-	+++
Gelatinase B	+++	+++	-	+++
Collagenase-3	-	-	-	+++
TIMP-1	+	+	+	+
TIMP-1	+	+	+	+

Induction of MMPs is mediated by a secreted factor.

The identity of the factor produced by epithelial cells that induces MMP gene expression in the metaplastic cells is not presently known. However, experiments have determined that the effect of co-culture on the metaplastic cells can be reproduced at least partially by

feeding them with medium conditioned by the epithelial cells, suggesting the secretion of a soluble MMP inducing factor by the epithelial cells. The factor is unlikely to be emmprin, as strong mRNA signals for it are obtained by PCR analysis of both epithelial cells and metaplastic cells. Time-course experiments using RNA synthesis inhibitors have determined that the induction of collagenase-3, stromelysin-1 and gelatinase B mRNAs in M-cells is not a post-transcriptional effect. Moreover, induction of these mRNAs is rapid, being detectable within 2h of the addition of stimulatory medium.

Stromelysin-1 gene regulation.

Cells from a metaplastic clone, BC1-M3, have been stably transfected with reporter gene plasmids in which transcription of the neo gene, which confers resistance to G418, is driven by a full-length (-1100 to + 8, with respect to transcription start site) stromelysin-1 promoter, and a CAT gene driven by either a full-length stromelysin-1 promoter or a stromelysin-1 promoter truncated to its TATA box. After some misleading results based on G418 resistance, it was determined by PCR analysis that the full-length promoter is insufficient to confer inducibility by E-cell-conditioned medium on the reporter gene (Table 2). Thus, the critical elements determining over-expression of the stromelysin-1 gene lie outside of this proximal promoter and upstream region. This region had previously been determined to mediate control of transcription by tumor-inducing agents and retinoids (Nicholson et al., 1989; Wasylyk et al., 1991).

Collagenase-3 gene regulation.

Quantitative PCR analysis indicated that collagenase-3 mRNA, like that of stromelysin-1, is up-regulated in metaplastic cells by conditioned medium from epithelial cells.

Two bacteriophage P1 clones were obtained by an initial commercial screening of a rat genomic DNA library by Genome Systems, Inc. containing the collagenase-3 gene. After considerable time spent analyzing the two clones, it was determined that neither of them contained genuine collagenase-3 sequences, and that the original PCR-based screening of the P1 library must have been flawed in some way. The company was informed and agreed to re-screen the library at no extra cost, using a new PCR primer set. A single P1 clone was obtained and verified by us to contain collagenase-3 proximal promoter sequences to ≥ 6kbp upstream of the transcription start site and the entire open-reading frame encoding the protein. This clone gives identical bands to BC1-derived genomic DNA in Southern blots of restriction enzyme digests probed with a proximal promoter-derived probe. A region of DNA spanning ~6kbp of DNA 5' to the transcription start site was examined for DNase hypersensitive sites induced in M-cells by exposure to E-cell conditioned medium. None was detected. Thus, either the up-regulation of collagenase-3 gene expression did not result from alterations in the chromatin structure of the upstream region, or the alterations were not detected as a result of insufficient assay

sensitivity e.g. a small proportion of the M-cells at any one time may have been induced by the E-cell conditioned medium.

Transient transfections in which 2000bp of collagenase-3 promoter and upstream sequences drove reporter genes demonstrated that this region was insufficient to mediate the E-cell-conditioned medium induction of collagenase-3 in M-cells (Table 2). Thus, the region responsible for upregulating MMP13 gene expression in M-cells in response to the E-cell factor is an enhancer either downstream or more than 2000bp upstream of the transcription start site.

Thus, a new approach is needed to identify the enhancer mediating the high level of expression of the three MMP genes in M-cells. To this end, we have begun constructing a reporter gene that includes the entire 80kbp of the rat collagenase-3 gene within the P1 clone. A cassette including a luciferase open reading frame is being used to replace part of the first exon. This will enable localization of the enhancer through gross deletion analysis, followed by finer deletion and mutation analysis, analogous to that originally proposed for the proximal promoter. The introduction of ET cloning (Zhang et al., 1998) into the laboratory has enabled this sort of manipulation of P1-size plasmids to be done.

Gelatinase B gene regulation.

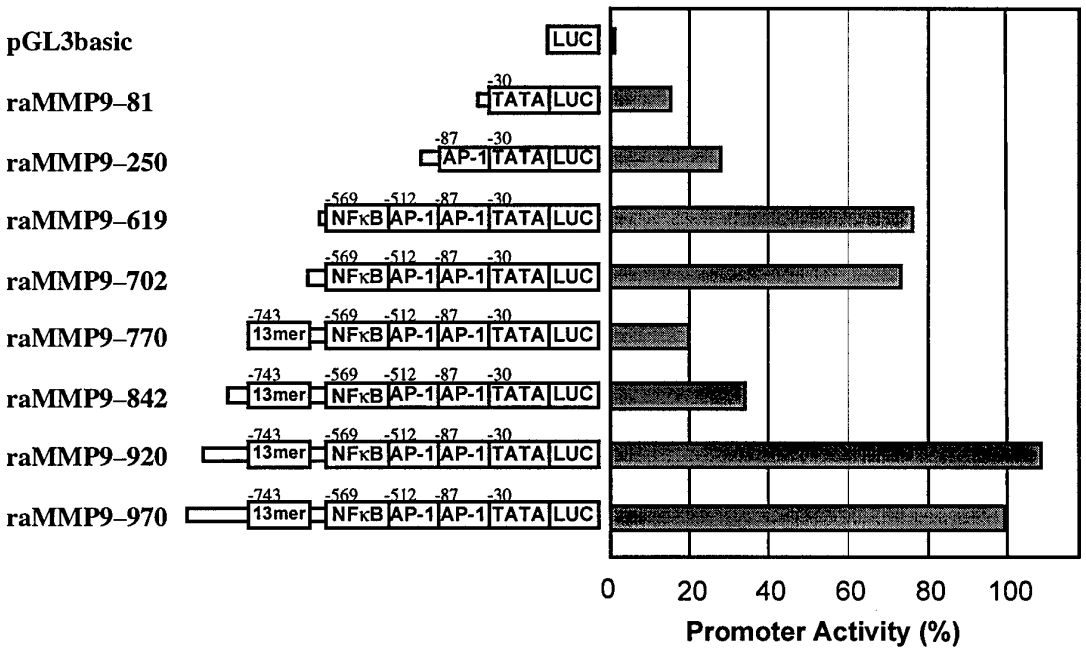
Gelatinase B, like stromelysin-1 and collagenase-3, is up-regulated in M-cells upon co-culture with E-cells. The rat gelatinase B promoter region was isolated by PCR using consensus primers corresponding to sequences conserved in the mouse and human genes. By transient transfection studies, it was found that, like stromelysin-1 and collagenase-3, the proximal promoter region of gelatinase B (764bp upstream of the transcription start site) was not sufficient to mimic up-regulation of the endogenous gene in M-cells in response to E-cell-conditioned medium (Table 2). The gelatinase B promoter sequence has subsequently been extended to 970bp upstream from the transcriptional start site by using inverse PCR to clone it.

Table 2. Summary of the role of upstream promoter regions in mediating high-level MMP gene expression in mammary carcinoma cells.

Upstream region	**Mediates Inducible Expression in M-cells**	**Mediates Constitutive Expression in E-cells**
Stromelysin-1 (-1100)	No	Not applicable
Gelatinase B (-970)	No	Yes
Collagenase-3 (-2000)	No	Not applicable

Unlike stromelysin-1 and collagenase-3, gelatinase B is produced constitutively by E-cells. By using transient transfection studies, we have found that the 970bp upstream region is sufficient to confer transcription in E-cells (Table 2). The cell/gene specificity was confirmed by transfection of E-cells with a reporter gene that was driven by the collagenase-3 upstream region, which produced low levels of transcription. Important elements for the constitutive gelatinase B transcription lie between positions -764 and -250 of the upstream region. This region includes binding sites for the transcription factors NFkB and AP1 which have been demonstrated previously to mediate the high level of MMP9 transcription seen in other tumor types (Sato and Seiki, 1993). In addition, a novel upstream element responsible for repressing transcription of the MMP9 gene has been identified. This region is currently being targeted by mutagenesis to define the critical bases that reduce the high levels of constitutive gelatinase B, using transient transfections. Moreover, an adjacent region can overcome this repression. When the

Figure 1. Luciferase reporter gene analysis determines that a narrow region containing the conserved 13mer repeats confers repressor activity on the promoter.



BC1-E2 cells were transfected with reporter genes in which transcription of the firefly luciferase open reading frame is driven by the full 970bp of the MMP9 promoter (-970) or3' truncations of this promoter (-920, -842, -770, -702, -619, -250, -81). A schematic representation of the reporter gene constructs and the positions of important transcription factor elements is shown at left. Luciferase activity was measured and plotted as a percentage of the full-length promoter activity on the right.

precise locations of these novel down- and up-regulatory sequences have been defined *in vitro*, their roles in cancer *in vivo* will be confirmed by the injection of stably transfected E-cells into animals and measuring the reporter gene activity in tumor lysates. Future projects will focus on the identity of the repressor factor and determine whether its upregulation in breast cancer cells is a feasible way of down-regulating MMP9 production.

Discussion

The identification of a paracrine factor made by breast cancer cells that is able induce MMP gene expression in other breast cancer cells is novel. We do not yet know much about this factor. However, it is unlikely to be emmprin, as both E-cells and M-cells have high levels of mRNA for emmprin (Guo et al., 1997), and it is unlikely to be one of the previously identified growth factors/cytokines that induce transcription of MMPs through their proximal promoter regions, as these have been demonstrated to be inadequate to mediate induced transcription of stromelysin-1, gelatinase B and collagenase-3 in M-cells. Thus, the factor may turn out to be a novel regulator of MMP production in breast cancer cells.

Because both epithelial cells and metaplastic cells are exposed to the epithelial cell-derived MMP inducing factor, but only the metaplastic cells actually express their MMP genes, there must be an inherent biochemical difference between the cells that underlies this. This could be that an appropriate receptor for the factor is absent in the epithelial cells but present in the metaplastic cells. This may explain the different responsiveness of the collagenase-3, stromelysin-1 and stromelysin-2 genes, but would require a different factor being present for the induction of the gelatinase B gene, which is constitutively active in the epithelial cells. Alternatively, it may be that epigenetic differences between the two cell types, perhaps in the chromatin structure or methylation status of the collagenase-3 and stromelysin-1 and -2 genes, make them unresponsive in the epithelial cells. Thus, two stages are required for a high level of MMP induction in these mammary carcinoma cells: (1) the attainment of inducibility, and (2) actual induction by the inducing factor secreted by the epithelial cells.

It is interesting that at least two mechanisms are responsible for high-level MMP production by breast cancer cells: one represented by the high constitutive level of expression of the gelatinase B gene in E-cells and the other by the high inducible level of expression of MMPs, including gelatinase B, in M-cells. This suggests that complete

control of MMP production targeted by future therapies may require complex mixtures of agents.

As far as technical approaches go, the degree of labor-intensiveness and time-consuming nature of generating the stably transfected cell lines lead us to use transient transfections as a preliminary screening process for determining the effects of promoter mutations on MMP gene expression. This enabled us to make up some time during the past year and to identify a region of the gelatinase B gene upstream region that is critical for its constitutive expression in E-cells, as well as the novel repressor region. The outcome of the data obtained from transiently transfected DNA will still require verification with stably transfected reporter genes and subsequent *in vivo* analysis.

(7) KEY RESEARCH ACCOMPLISHMENTS

- Determination of the absolute number of MMP mRNA molecules in the mammary carcinoma cells, both *in vivo* and *in vitro*, allowing us to determine that the mRNAs of collagenase-3, stromelysin-1 and gelatinase B are major gene products in BC1 cells and tumors, being in the top 2% of expressed genes.
- Identification of metaplastic cells as being the major source of collagenase-3, stromelysin-1 and stromelysin-2 in BC1, whereas gelatinase B is produced by both the epithelial cells and the metaplastic cells.
- Demonstration that the metaplastic mammary carcinoma cells are the result of an epithelial-mesenchymal transition
- Determination that an epithelial cell-derived MMP-inducing factor is released in a soluble form.
- Acquisition and analysis of a P1 clone containing the rat collagenase-3 gene.
- Cloning and sequencing of the rat gelatinase B and collagenase-3 promoter and upstream region.
- Determination that the collagenase-3, stromelysin-1 and stromelysin-2 genes are differentially induced in epithelial and metaplastic cells.
- Demonstration that the promoter upstream regions of the stromelysin-1, gelatinase B and collagenase-3 genes are insufficient to confer inducibility to epithelial cell conditioned-medium in metaplastic cells.
- Demonstration that the upstream promoter upstream region of the gelatinase B gene is sufficient to confer a high level of transcription and that elements critical for this effect lie between 619 and 250bp upstream from
- Demonstration of a repressor region between 702 and 764bp upstream from the transcription start site.

(8) REPORTABLE OUTCOMES

Publications:

Martorana AM, Zheng G, Crowe TC, O'Grady RL, Lyons JG.
Epithelial cells upregulate matrix metalloproteinases in cells within the same mammary carcinoma that have undergone an epithelial-mesenchymal transition. Cancer Res. 1998; 58:4970-4979.

Martorana AM, Zheng G, Springall F, Iland HJ, O'Grady RL, Lyons JG. Absolute quantitation of specific mRNAs in cell and tissue samples by comparative PCR. BioTechniques 1999; 27:136-144.

Presentations:

Martorana, A. M., Zheng, G., Springall, F., Iland, H. J., O'Grady, R. L. and Lyons, J. G.
"MMP induction in breast cancer cells via interclonal co-operativity."
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Martorana A.M., Zheng G., Shoebridge, G., Lyons J.G.
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Beatson International Conference on Invasion and Metastasis, Glasgow, Scotland, 1999.

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Zheng G., Martorana A.M., Lyons, J.G.
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Min, D., Moore, A., Breit, S., and Lyons, J.G.
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J.G. Lyons

"MMP gene regulation and the epithelial-mesenchymal transition."
Era of Hope D.O.D. Breast Cancer Research Program Meeting, 2000

Nucleotide Sequences:

Genbank accession AF148065: Rattus norvegicus matrix metalloproteinase 9 gene, promoter.

Genbank accession AF148064: Homo sapiens matrix metalloproteinase 9 gene, exons 1, 2, intron 1.

Scholarship:

NHMRC Dora Lush Postgraduate Award was awarded to Guoping Zheng, a graduate student previously supported by this grant.

PhD Thesis:

Danqing Min, a graduate student supported, in part, by this grant, was awarded her PhD in April, 2001. The thesis was entitled:
"Molecular mechanisms of regulating the expression of matrix metalloproteinases –2 and -9."

Funding Applied for Based on this Work:

Funding has been sought from the Australian National Health and Medical Research Council and other Australian funding agencies for the following projects that have grown out of the present one: "Epithelial-mesenchymal transition and matrix metalloproteinase production in carcinomas"; "The transcriptional repressor operating through a novel element in the MMP9 gene".

(9) CONCLUSIONS

We have determined for the first time the actual number of MMP mRNA molecules in mammary carcinoma cells and tumors. This has allowed us to determine that they are, indeed, present at high absolute levels, and not just higher relative to the signal in some other source of mRNA, and will allow other researchers to compare levels in their cancer samples with ours, if they also decide to undertake absolute quantitation of MMP mRNAs. Eventually, a picture may develop as to what "high" levels of mRNAs actually are for these MMPs and whether they correlate with the *in vivo* behavior of the tumors. The transfection of reporter genes driven by MMP promoters has enabled us to determine that their proximal promoters and upstream regions are not responsible for mediating the high level of expression induced in metaplastic cells. These regions are the ones most commonly examined by biologists for regulatory functions. On the other hand, the high levels of gelatinase B made by the epithelial cells are mediated by the upstream region. Thus, there are at least two mechanisms responsible for high-level MMP production by breast cancer cells. This suggests that complete control of MMP production targeted by future therapies may require complex mixtures of agents. The high time-consumption of this approach has lead us to explore the possibility of using transient transfections in the future as a means of a first screening of promoter mutations. However, the critical mutations will need to be verified in stably transfected cells *in vitro* and *in vivo*, to ensure the relevance of the finding to gene regulation in actual mammary carcinomas. The determination that the epithelial-derived MMP-inducing factor is a secreted product opens up the possibilities of future studies aimed at identifying it and determining whether it may represent a therapeutic target. The finding that an EMT is associated with the ability to produce high levels of MMPs leads us to two questions regarding its importance in the processes of invasion and metastasis: (1) is an EMT actually necessary or sufficient to confer MMP gene inducibility on carcinoma cells? (2) how widespread are EMTs in human breast cancer? The latter question has been addressed by Wargotz and colleagues (Wargotz et al., 1989a, b). However, their definitions of carcinomas in this category required histological evidence of a large proportion of the tumor mass. Considering our observation *in vitro* that the highest total MMP production occurs when the metaplastic component of the carcinoma cell population is only 10%, it would be interesting to know the proportion of breast carcinomas in which a small proportion of the total mass has undergone an EMT and what pathological behavior this confers on the tumors. New markers that allow us to distinguish between metaplastic carcinoma cells and host stromal cells may be required for this purpose. Thus, the results obtained so far should be useful both to ourselves, to bring the project to a successful conclusion, and to breast cancer researchers elsewhere, to incorporate into their own knowledge of the molecular mechanisms underlying tumor behavior. A direct clinical application of the

results is probably some way off, although the method for absolute quantitation of mRNAs could find a use in diagnostic applications.

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(13) PUBLICATIONS AND PERSONNEL

Publications in refereed journals arising from this grant:

Martorana AM, Zheng G, Crowe TC, O'Grady RL, Lyons JG.
"Epithelial cells upregulate matrix metalloproteinases in cells within the same mammary carcinoma that have undergone an epithelial-mesenchymal transition."
Cancer Res. 1998; 58:4970-4979.

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"Absolute quantitation of specific mRNAs in cell and tissue samples by comparative PCR."
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Meeting abstracts:

Martorana, A. M., Zheng, G., Springall, F., Iland, H. J., O'Grady, R. L. and Lyons, J. G.
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S. Supple (Technician)

J. Ayling (Technician)

[CANCER RESEARCH 58, 4970-4979, November 1, 1998]

Epithelial Cells Up-Regulate Matrix Metalloproteinases in Cells within the Same Mammary Carcinoma That Have Undergone an Epithelial-Mesenchymal Transition

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ABSTRACT

A metastatic rat mammary carcinoma cell line, BC1, contains cells that have retained epithelial differentiation characteristics and metaplastic cells that have undergone an epithelial-mesenchymal transition. These two subpopulations cooperate to degrade collagen. We have used novel PCR assays to quantitate, for the first time, absolute levels of the mRNAs encoding matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in cell and tumor samples. BC1 tumors expressed high levels of the *collagenase-3, TIMP-2, stromelysin-1,* and *gelatinase B* genes and low levels of the *stromelysin-2* and *TIMP-1* genes. This pattern of expression was repeated in cultures of BC1 and cultures containing mixed clones of epithelial cells and metaplastic cells. In both BC1 and the biclonal cultures, metaplastic cells were the main source of collagenase-3, stromelysin-1 and stromelysin-2, whereas TIMPs were equally distributed and epithelial cells were the main source of gelatinase B. High levels of all four MMP mRNAs in metaplastic cells were dependent on coculture with epithelial cells, suggesting the production of an inducing factor by the epithelial cells. In contrast, gelatinase B mRNA was produced at a high level by epithelial cells in the absence of metaplastic cells. TIMP-2 mRNA was abundant in both subpopulations grown alone and did not change substantially upon coculture. Thus, the interclonal cooperativity to degrade collagen in BC1 cells required the induction of MMPs in metaplastic cells by epithelial cells. Interclonal cooperativity may be important to the progression of neoplastic tumors, a feature of which is phenotypic heterogeneity.

INTRODUCTION

MMPs[3] are a family of enzymes that are believed to mediate much of the extracellular matrix degradation that occurs in developmental and pathological processes, including tumor invasion and metastasis (1, 2). They include the interstitial collagenases, the gelatinases, and the stromelysins. Of particular importance is the ability of the interstitial collagenases to initiate degradation of interstitial collagens, a property unique to these enzymes. After the initial cleavage by an interstitial collagenase, other proteinases can degrade the partially hydrolyzed collagen into oligopeptides; the gelatinases are particularly efficient at this, because of their high affinity for denatured collagens, and can cooperate with interstitial collagenases in the destruction of collagens.

Destruction of extracellular matrix components occurs around neoplastic tumors and is required for invasion and metastasis (3, 4). An imbalance between MMPs and their specific inhibitors, the TIMPs, in favor of the MMPs has been proposed as a mechanism for mediating this destruction and has been observed in some cases (2, 5). Both direct overproduction of MMPs by the neoplastic cells and induction of MMPs in the tumor-associated stroma have been implicated as causes of this imbalance (6-11). Expression of the *collagenase-3* gene, for example, is associated with breast cancer cells (6). Overproduction of MMPs in some cells *in vitro* can be induced by cancer-associated cytokines and by aberrant expression of oncogenic signal transduction factor and transcription factor genes (1, 12-14).

Phenotypic heterogeneity is a feature of cancer cells. Contributing to it in carcinomas is metaplastic transformation, in which the epithelial characteristics of the neoplastic cells are lost and which is associated with a more aggressive behavior (15-20). The presence of clones of different phenotypes within the neoplastic cell population gives rise to the opportunity for interactions between them, which could alter the overall behavior of the tumor. Thus, clonal populations of neoplastic cells could cooperate to exhibit a more aggressive phenotype in coexistence than when present alone. In one such instance of interclonal cooperativity, the potential of a biphenotypic mammary carcinoma cell line to degrade collagen is enhanced when both subpopulations of cells are present (21). Using a novel assay that permits absolute quantitation of specific mRNAs, we have for the first time been able to compare absolute mRNA levels of different MMPs and TIMPs with one another in tumors and cultured mammary carcinoma cells. The results demonstrate that the induction of *MMP* gene expression in cancer cells that have undergone an epithelial-mesenchymal transition by cells that have retained their epithelial differentiation characteristics underlies this cooperativity to degrade collagen.

MATERIALS AND METHODS

Cell Cultures and Tumors. BC1 and its derivative clonal cell lines, E2 and M3, were cultured under continuously serum-free conditions as described (21, 22). Basal media and other culture chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). For RNA extraction and immunoblot analysis, the cell lines were plated in medium containing 20% self-conditioned medium (v/v), at a plating ratio of 1:20 in either 75 or 150 cm^2 Corning tissue culture flasks, and grown to confluence. To obtain cocultures of E2 and M3, the cells were plated together at a ratio of 4:1, a ratio at which production of collagenolytic activity is maximal (21). Cultures were fed with fresh medium, supplemented with 20% self-conditioned medium, 2 days prior to the extraction of RNA. At the time of RNA extraction, conditioned medium was collected from each cell line, clarified by centrifugation, and stored at -20°C until assayed for collagenolytic activity by the method of Nethery *et al.* (23). Except for experiments in which epithelial and metaplastic cells were isolated by differential trypsinization, cells were lysed for RNA extraction directly in their flasks. To separate metaplastic cells from epithelial cells, 150-cm^2 flasks were rinsed gently two times with medium, followed by the addition of 5 ml of a solution of 0.1% trypsin, 0.02% EDTA in PBS. The cells were observed continuously by phase contrast microscopy to determine when the metaplastic cells were detached (~4.5 min). At this time, the metaplastic cell fraction was collected, and an equal volume of 0.1% soybean trypsin inhibitor in PBS was added concurrently to the metaplastic cell fraction and to the flask containing attached epithelial cells. In this way, both the metaplastic and epithelial cell fractions were trypsinized for the same period of time. While the metaplastic

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[3] The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

cells were pelleted by centrifugation, the flask containing epithelial cells was rinsed two times with PBS to remove residual metaplastic cells, leaving behind firmly attached epithelial cells. The two cell fractions were then processed for the extraction of RNA, by the addition of cell-lysis solution to the metaplastic cell pellet, and to the epithelial cells still attached to the flask. To determine the number of cells from which RNA was extracted, a representative flask was trypsinized until all of the cells were detached and hemocytometer counts were performed on both the metaplastic and epithelial cell fractions, as appropriate.

For tumorigenesis, BC1 cells were grown to confluence, harvested by trypsinization, and washed twice with PBS. The cell pellet was resuspended in PBS at a concentration of 1×10^7 cells/ml. Cells (0.5×10^6) in 50μl were injected into the right footpad of each female Dark Agouti rat, 7 weeks of age (Animal Resources Center, Perth, Australia). Tumors were allowed to grow for a period of 40 days before harvesting. Upon harvesting, both the left and right feet were amputated and weighed. The mass of the right footpad tumor was determined to be the difference in mass between the left and right feet. The footpad tumor was excised and cut into one-third and two-third portions, which were also weighed. The one-third portion was fixed in 10% formalin for histological analysis, and the two-thirds portion was stored in liquid nitrogen for the extraction of RNA. Formalin-fixed specimens were embedded in paraffin, cut into 5-μm sections, and stained with H&E. The number of cells from which tumor RNA was extracted was calculated assuming 950×10^3 cells/mg tissue, a figure determined empirically from the packed cell volume of trypsinized cultured cells.

The right and left popliteal lymph nodes were removed by blunt dissecting along the midline of the biceps femoris muscle, which encloses the node, and detaching the exposed lymph node from its supporting connective tissue. The right and left lymph nodes were weighed, and the right lymph node was dissected into one-third and two-third portions. The one-third portion was fixed in 10% formalin for histological analysis, and the two-thirds portion was placed in Ham's F12/DME (1:1), with 100 units/ml penicillin, for the preparation of primary cultures. The left popliteal lymph node was also fixed in 10% formalin for histological analysis. The weight of the lymph node metastasis in the right lymph node was determined as the difference in mass between the right and left lymph nodes.

Cell lines were obtained from lymph node metastases by cutting the two-thirds portion of the node into small (~1 mm^3) pieces and placing them in a flask of Ham's F12/DME (1:1), supplemented with 10% FCS (Cytosystems, Castle Hill, Australia). Once cell lines were established, they were maintained under serum-free conditions as for BC1 (22).

Quantitative PCR Assays. Total RNA was extracted from cultured cells and tumors according to the method of Chomczynski and Sacchi (24), and absolute quantitation of MMP and TIMP mRNAs was done by competitive PCR[4]. Tumors, kept frozen in dry ice, were pulverized using a mortar and pestle prior to the addition of tissue lysis solution.

Immunoblot Analysis. Culture media samples were concentrated by precipitation with 60% saturated ammonium sulfate, which precipitates MMPs, but not BSA, electrophoresed according to Laemmli (25) in 10% polyacrylamide gels under nonreducing conditions and transferred to polyvinylidene difluoride membranes. Cell cultures for immunoblot analysis were washed twice with protein-free culture medium and lysed with sample buffer containing 0.1M DTT. Membranes were probed for collagenase-3 with the mouse monoclonal antibody, CoBCl-IID1 (26), for stromelysin-1 with a rabbit polyclonal antibody raised against a COOH-terminal peptide (27), for keratin with a rabbit anti-pan-keratin polyclonal antibody (Dako), for E-cadherin with a monoclonal antibody (Transduction Laboratories), and vimentin with a monoclonal antibody (Zymed), according to the suppliers' instructions. Immunoreactive bands were detected using horseradish peroxidase-conjugated second antibodies and ECL chemiluminescence (Amersham), according to the manufacturer's instructions.

RESULTS

Metastatic Behavior of BC1 Subpopulations. The cell line, BC1, was injected into the right footpads of six Dark Agouti rats. The primary tumors grew rapidly and invaded the adjacent lumbricales muscles (Table 1; Fig. 1*A*). Five of the six rats developed markedly enlarged right popliteal lymph nodes (Table 1). Histological examination showed that all six rats had lymph node metastases (Fig. 1, *B* and *C*). The enlarged lymph nodes varied with respect to the degree to which the metastasis had replaced the original architecture and lymphatic tissue. Generally, tumor cells could be seen in the subcapsular sinuses, where these were still intact, and also in the medullary sinuses. In some of the enlarged lymph nodes, entire sections of the node had been replaced by tumor cells, indicating that BC1 cells were not only capable of metastasizing via the afferent lymphatic vessel but were also able to grow within the node itself.

BC1 is composed of two morphologically distinct populations of cells: epithelial cells, which have retained epithelial characteristics, including the presence of microvilli, tight junctions, and colony-style growth; and metaplastic cells, which have not (21). Immunoblot analysis demonstrates that the production of keratin and E-cadherin, proteins characteristic of epithelial tissues, is retained by epithelial cells, but not by metaplastic cells (Fig. 2*A*, *Lanes 1-4*), suggesting that metaplastic cells are the result of an epithelial-mesenchymal transition (28). Both types of cells produce vimentin, which was more abundant in metaplastic cells than in epithelial cells (Fig. 2A, Lanes 5 and 6).

To determine whether both epithelial cells and metaplastic cells of BC1 contained subpopulations that were potentially metastatic, cell lines were established from the popliteal lymph node metastases of five of the six rats; there was insufficient material to establish a culture from the lymph node from one of the six rats. Overall, both epithelial and metaplastic cells could metastasize, as evident from their presence in at least some of these cell lines (Fig. 1, *D-H*). However, not all cell lines from metastases retained both cell types. Two cell lines, 2LN and 3LN, resembled the parental cell line in that they contained morphologically identifiable populations of both epithelial and metaplastic cells (Fig. 1, *D* and *E*). 4LN had no identifiable metaplastic cells during early passages, but metaplastic cells were noticed later among the epithelial cells, suggesting either an expansion of a small number of metaplastic cells from the primary culture or the generation of metaplastic cells from precursors within the culture (Fig. 1*F*). Neither 5LN nor 6LN contained typical spindle-shaped metaplastic cells that could be removed from the flask with less than 4 min of trypsin treatment (Fig. 1, *G* and *H*). Thus, epithelial cells metastasized to the popliteal lymph node and grew there, regardless of whether metaplastic cells were also present in the lymph node. When the 6LN cultures, which were uniformly epithelial, were subjected to a second round of footpad injection and lymph node harvest, cultures of the lymph nodes yielded cells still composed only of the epithelial morphology. Similarly, when clonal metaplastic cells (M3) were injected into the footpad, cultures of the draining lymph nodes harvested after 6 weeks showed that two of six lymph nodes had only viable M3 cells in them. No cell lines could be established from

Table 1 *Tumor and lymph node size in BC1 tumor-bearing rats*

	Mass (g)	
Rat no.	Primary tumor	Metastasis
1	0.23	0.032
2	0.45	0.17
3	0.47	0.51
4	0.41	0.31
5	0.46	0.32
6	0.55	0.19
Mean + SD	0.43 ± 0.11	0.26 ± 0.16

For each rat, 0.5×10^6 BC1 cells were injected into the footpad and allowed to grow for 40 days, and the masses of the tumors and lymph nodes were determined on an electronic balance, as described in "Materials and Methods."

[4] A.M. Martorana, G. Zheng, F. Springall, H. J. Iland, R. L. O'Grady, and J. G. Lyons. Absolute quantitation of specific mRNAs in cell and tissue samples incorporating an external standard, submitted for publication.

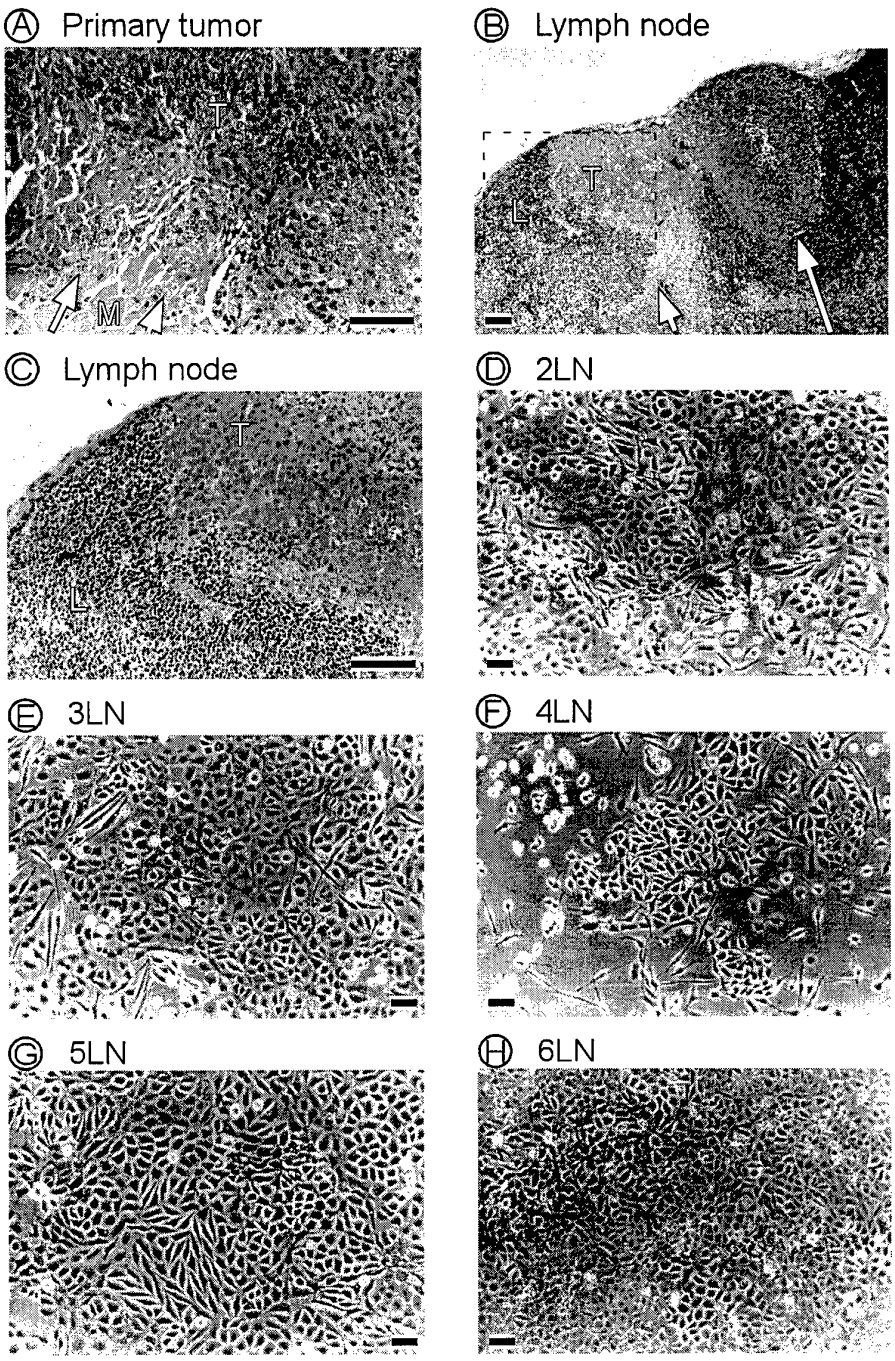

Fig. 1. Behavior of BC1 *in vivo*. Tumors were grown in the footpads of six syngeneic rats by injection with 0.5×10^6 BC1 cells and allowed to grow for 40 days. Samples of the primary tumor and lymph nodes were taken for histological analysis, RNA extraction, and primary culture. H&E-stained sections of primary tumor (*A*) and draining lymph node (*B* and *C*) are shown. The area *boxed* in *B* is shown at a higher magnification in *C*. Areas of tumor (*T*), muscle (*M*) and lymph node (*L*) are indicated. *Arrows*, invasive edges of tumors. The lymph nodes of rats numbers 2-6 were excised and used to establish cell lines 2LN, 3LN, 4LN, 5LN, and 6LN, depicted in phase contrast photomicrographs *D-H*, respectively. Cells exhibiting the spindle shape characteristic of the metaplastic cell phenotype are evident in panels *D-F* but not in *G* and *H*. *Bar*, 50μm

control lymph nodes under the conditions used for the BCl-derived cell lines. In both 6LN and M3, no evidence of an *in vivo* transition from metaplastic phenotype to epithelial phenotype or *vice versa* was observed. Thus, although epithelial and metaplastic cells may undergo a phenotypic transition *in vivo*, they appear to be able to reach the draining lymph nodes from the primary tumor without doing so.

Complementation of *MMP* and *TIMP* Gene Expression Is Not Sufficient to Generate Collagen-degrading Activity. Cultures of epithelial and metaplastic cells that are grown in isolation do not make significant levels of collagenolytic activity, whereas in coculture they make high levels (21). It was possible that complementation of gene expression could account for the acquisition of collagenolytic activity during coculture of epithelial and metaplastic cells, given the following scenario. The epithelial cells produce no interstitial collagenase, which is an absolute requirement for collagen degradation, but they produce an excess of MMPs (mainly gelatinase B) over TIMPs; the metaplastic cells, however, produce collagenase-3, but because their production of TIMPs exceeds their production of total MMPs, they are unable to produce a net collagen degrading activity. When the two media are mixed, the total level of MMPs exceeds the total level of TIMPs, and sufficient free collagenase-3 is available to initiate collagen degradation. This possibility was examined by mixing media

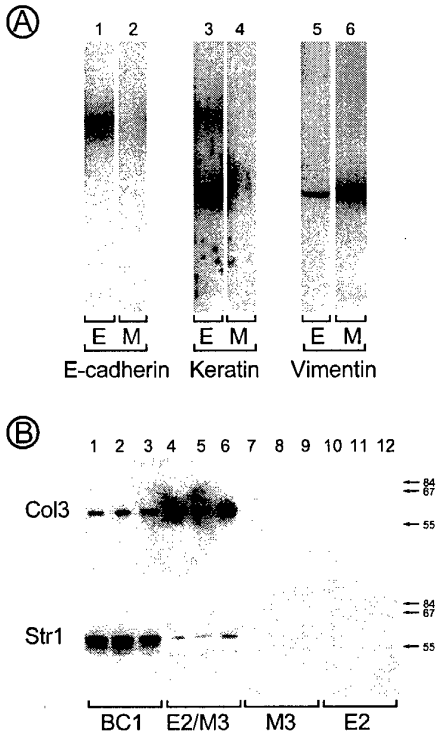

Fig. 2. Immunoblot analysis of epithelial differentiation markers and MMP production in BC1 and clonal derivatives. *A*, immunoblots of lysates of E2 or M3 cells, equivalent to 5 × 10^3 cells per lane, were probed with antibodies to E-cadherin *(Lanes 1* and *2)*, pan-keratin *(Lanes 3* and *4)*, and vimentin *(Lanes 5* and *6)* and detected by chemiluminescence. *B*, immunoblots of culture media from three separate platings of BC1 *(Lanes 1-3)*, cocultures of E2 and M3 *(Lanes 4-6)*, M3 grown alone *(Lanes 7-9)*, and E2 grown alone *(Lanes 10-12)* were probed for collagenase-3 and stromelysin-1 with specific antibodies. The positions of molecular weight markers (in thousands) are shown to the *right* of the membranes.

conditioned by each of the two subpopulations grown in isolation at a ratio of 4:1 (epithelial cell medium:metaplastic cell medium) and assaying it for collagenolytic activity. However, in three separate experiments, no net collagenolytic activity was generated. Thus, complementation of secreted MMPs was not normally sufficient to account for the increase in collagen degradation caused by mixing epithelial cells and metaplastic cells in culture.

Expression of *MMP* and *TIMP* Genes in BC1 Tumors *in Vivo* and Cultures *in Vitro*. To determine the levels of expression of *MMP* and *TIMP* genes *in vivo* in BC1 tumors, steady-state mRNA levels for collagenase-3, gelatinase B, stromelysin-1, stromelysin-2, TIMP-1, and TIMP-2 were quantitated by competitive PCR (Fig. 3A). Collagenase-3 and TIMP-2 mRNA were present at very high levels (>100 copies per cell), and gelatinase B and stromelysin-1 mRNAs were present at intermediate levels (20-100 copies per cell), whereas stromelysin-2 and TIMP-1 mRNAs were present at the lowest levels (<20 copies/cell).

A comparison of the levels of expression of *MMP* and *TIMP* genes in BC1 cells in culture with the levels in BC1 tumors revealed that the pattern of expression *in vivo* resembled that of BC1 cells *in vitro* (Fig. 3, A and B). Collagenase-3 and TIMP-2 mRNAs were present at very high levels in the cultured cells. TIMP-1 and stromelysin-2 mRNAs were present at very low levels *in vitro*. As in the tumors *in vivo*, gelatinase B and stromelysin-1 mRNAs also were present *in vitro*, although at relatively higher levels, comparable to those of collagenase-3 and TIMP-2 mRNAs.

E2 and M3 are clonal derivatives of BC1 that are representative of the epithelial and metaplastic phenotypes, respectively (21). To aid in the determination of which cells produce MMPs and TIMPs, E2 and M3 were cultured together, and the MMP and TIMP mRNA levels were measured by competitive PCR (Fig. 3*C*). The pattern of expression of *MMP* and *TIMP* genes in E2/M3 cocultures was similar to that seen in cultures of BC1. Collagenase-3 and TIMP-2 mRNA levels were high, with at least 95 copies/cell, whereas TIMP-1 and stromelysin-2 levels were consistently low, with the highest values measured at 16 and 23 copies/cell, respectively. Compared with the mRNA levels measured in BC1, gelatinase B mRNA was present in E2/M3 cocultures in somewhat higher quantities, with at least 540 copies/cell, whereas stromelysin-1 levels were somewhat lower, with the highest measured value at 60 copies/cell.

The increase in collagenolytic activity generated by mixing E2 cells with M3 cells (21) was reflected in an increase in secreted collagenase-3 and stromelysin-1 proteins, as detected by immunoblotting of

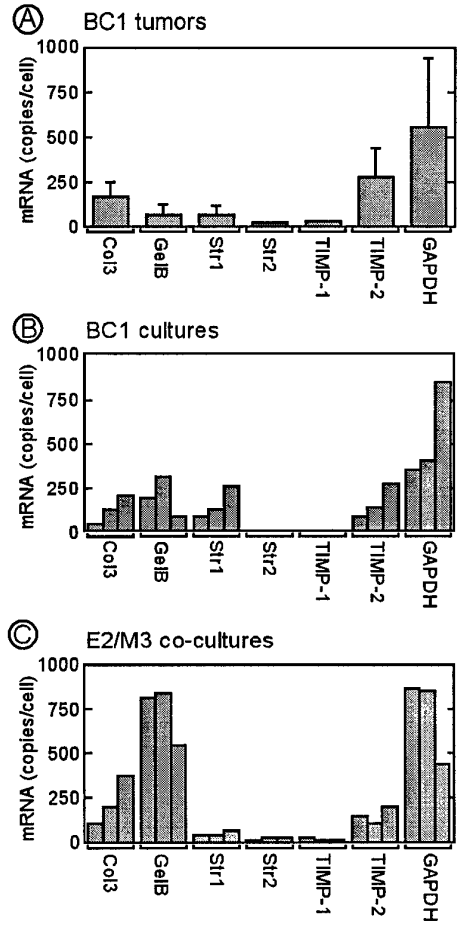

Fig. 3. *MMP* and *TIMP* gene expression in BC1 tumors *in vivo*, the BC1 cell line, and E2/M3 cocultures, cDNA was made from RNA that had been isolated from BC1-derived tumors and cells in the presence of the external standard APL1 RNA. Competitive PCR assays were used to measure the cDNA levels specific for collagenase-3 *(Col3)*, gelatinase B *(GelB)*, stromelysin-1 *(Str1)*, stromelysin-2 *(Str2)*, TIMP-1, TIMP-2, and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* in each of the total cDNA samples, and the values were corrected using the APL1 value and the standard discrepancies between expected and measured values, as described[4]. The corrected mRNA values, expressed as the number of copies per cell, are shown for BC1 tumors (*A*), BC1 cultures (*B*), and E2/M3 cocultures (*C*). The tumor values are the means of six individual tumors; *bars*, SD. Each *column* in the BC1 and E2/M3 graphs represents one of three individual experiments.

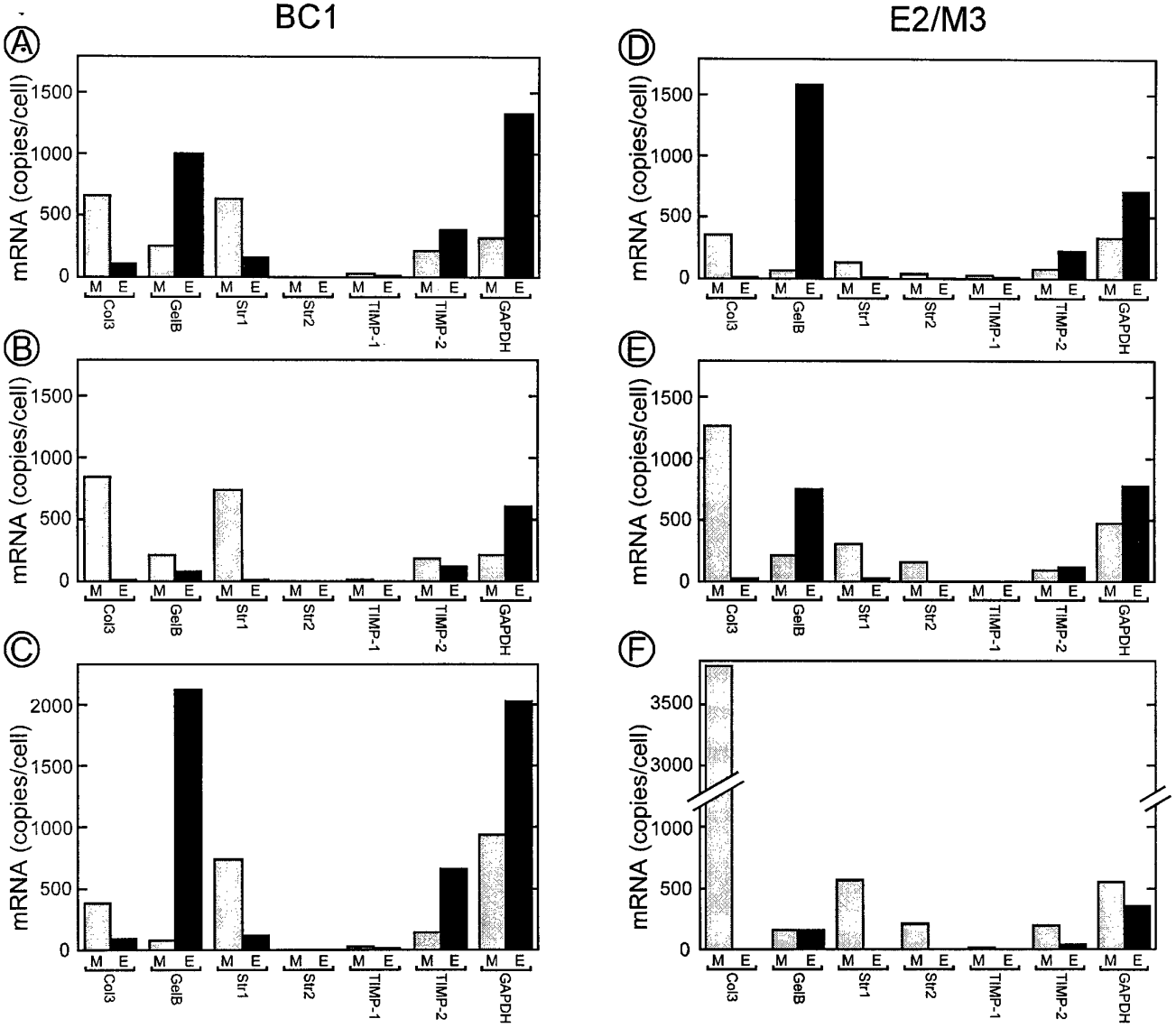

Fig. 4. Cellular origin of *MMP* and *TIMP* gene expression in BC1 and E2/M3 cocultures. Cultures of BC1 and E2/M3 cocultures were grown to confluence, and epithelial and metaplastic cell fractions were prepared from each by differential trypsinization. Competitive PCR assays were used to measure the mRNA levels for collagenase-3 *(Col3)*, gelatinase B *(GelB)*, stromelysin-1 *(Str1)*, stromelysin-2 *(Str2)*, TIMP-1, TIMP-2, and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* in each metaplastic cell fraction (*M*) and epithelial cell fraction (*E*). *A-C*, values of three individual experiments for BC1; *D-F*, values for three independent experiments for E2/M3.

culture supematants. Neither E2 nor M3 cultured alone consistently produced detectable collagenase-3 and stromelysin-1, whereas E2/M3 cocultures did so (Fig. 2*B*, compare *Lanes 4-6* with *Lanes 7-12)*, as did BC1 *(Lanes 1-3)*. Visualization of nonspecific bands by staining the membranes in Fig. 2*B* with Ponceau S prior to immunodetection demonstrated equivalent protein loadings (data not shown).

Cellular Origin of MMPs and TIMPs in BC1. To identify which population of cells in BC1 produces the MMPs and TIMPs, separate fractions of epithelial and metaplastic cells were prepared from BC1 by differential trypsinization. Epithelial and metaplastic cells differ with respect to their susceptibility to detachment from culture vessel surfaces by trypsin, to the extent that separate fractions of each may be prepared during the course of trypsinization of BC1 (21). Three separate differential trypsinization experiments were performed, and the mRNA levels of the MMPs and TIMPs were measured in the epithelial and metaplastic cell fractions of each by competitive PCR (Fig. 4, *A-C*). In all three replicates, metaplastic cells were the major source of collagenase-3 in BC1, with the number of mRNA transcripts per cell always measuring >380. In comparison, the number of collagenase-3 transcripts per epithelial cell was never >100, with the lowest measurement made at 12 copies/cell. In addition to providing most of the collagenase-3 mRNA in BC1, metaplastic cells were the major source of stromelysin-1 mRNA. The measured levels were always >600 copies per metaplastic cell, whereas the highest number of transcripts per epithelial cell was 150, and the lowest value was measured at 10 copies per epithelial cell. In two of the three replicates, epithelial cells produced most of the gelatinase B, containing at least 1000 copies of mRNA per cell, consistent with immunolocalization studies of the protein (26). Although the third replicate measured just 82 copies of gelatinase B per epithelial cell, the metaplastic cells in

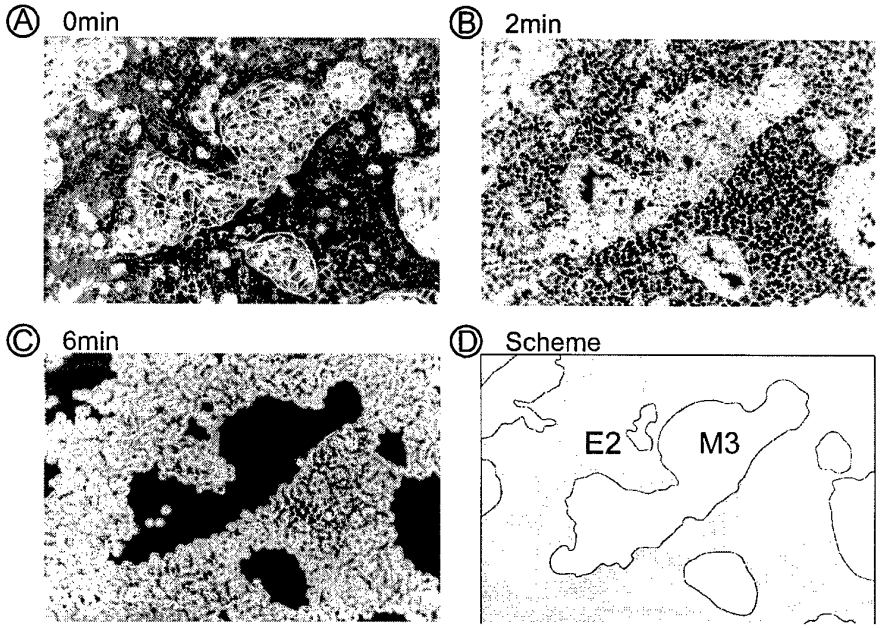

Fig. 5. E2 and M3 can be isolated from E2/M3 cocultures as separate fractions, using differential trypsinization. E2 and M3 cells were plated in the same flask at a ratio of 4:1 and allowed to grow to confluence. The coculture was rinsed once with F12/DME and treated at room temperature with a solution of 0.1% trypsin, 0.02% EDTA in PBS. Phase contrast photomicrographs of a typical field were taken. *A*, the E2/M3 coculture just prior to trypsinization. *B*, the same field of the culture after 2 min of trypsinization. *C*, the E2/M3 coculture after 6 min of trypsinization and gentle agitation to dislodge and remove detaching M3 cells. The E2 cells required an additional 5-6 min to detach. In *D*, the locations of E2 and M3 colonies are identified schematically.

BC1 never produced >240 copies/cell. TIMP-2 mRNA was found to be abundant in all epithelial and metaplastic cell fractions, with either cell fraction producing at least 120 copies of transcript per cell. TIMP-1 mRNA levels were consistently lower than TIMP-2 levels in both epithelial and metaplastic cell fractions, with the highest value measured in metaplastic cells at 18 copies/cell. The level of stromelysin-2 mRNA in whole BC1 and in any of the three epithelial cell fractions was below the limit of detection of the assay. However, it was detected in two of the three metaplastic cell fractions at 0.6 and 0.7 copies/cell, respectively, suggesting that stromelysin-2 is present in cultures of BC1, albeit at very low levels, and definitely not in all cells.

To determine whether the cellular origin of MMPs and TIMPs in E2/M3 cocultures was the same as that in BC1, separate E2 and M3 cell fractions were prepared from cocultures by differential trypsinization. Fig. 5 demonstrates that E2 and M3 can be isolated from E2/M3 cocultures in a manner analogous to that used for isolating epithelial and metaplastic cells from BC1. Three separate differential trypsinization experiments were performed, and the mRNA levels of the MMPs and TIMPs were measured in the E2 and M3 cell fractions of each by competitive PCR (Fig. 4, *D-F*). In all three replicates, the M3 cell fraction was the major source of collagenase-3 mRNA with the number of mRNA copies per cell measuring >350 and the highest value measured at 3800. In contrast, the level of mRNA transcripts for collagenase-3 in the E2 cell fraction was never >30 copies/cell. The M3 cell fraction was also the major source of stromelysin-1 and stromelysin-2 mRNA in all three replicates, with the number of transcripts per cell measuring at least 125 and 42, respectively. All three E2 cell fractions contained very low amounts of the stromelysin-1 and stromelysin-2 mRNAs, the highest values measuring at 5 and 4 copies/cell, respectively. In two of three replicates, the E2 cell fraction provided most of the gelatinase B mRNA copies, containing at least 750 copies/cell. Whereas the third E2 cell fraction contained just 165 copies per cell, the level of gelatinase mRNA in all three M3 cell fractions never exceeded 210 copies/cell, and the lowest value was measured at 65 copies/cell. TIMP-2 mRNA was present in both of the E2 and M3 cell fractions with at least 40 copies/cell in each, whereas TIMP-1 was present once more at lower levels, with the measured values in the E2 and M3 cell fractions ranging from 0.1 to 20 copies/cell.

Overall, the metaplastic cell fractions of BC1 and E2/M3 cocultures contained most of the collagenase-3, stromelysin-1, and stromelysin-2 mRNAs, whereas very high levels of gelatinase B mRNA could be produced only by the epithelial cell fractions. Both cell fractions contained TIMP-2 mRNA, at a high level, and TIMP-1 mRNA, albeit at a lower level.

Regulation of MMP and TIMP Gene Expression in Epithelial Cells and Metaplastic Cells during Coculture. Epithelial and metaplastic cells cultured in isolation do not produce collagenolytic activity in their culture medium, whereas in coculture they are able to do so (21). E2 and M3 cells in coculture are similar to the epithelial and metaplastic cells in BC1 cultures, with respect to their morphologies, their susceptibilities to detachment by trypsin, and the profile of MMPs and TIMPs that they produce. Thus, E2 and M3 were used for understanding the interactions between epithelial and metaplastic cells within BC1 that lead to a production of collagenolytic activity.

To determine whether the levels of MMP and TIMP mRNAs in epithelial cells could be regulated by metaplastic cells, the MMP and TIMP mRNA levels in E2 cells were measured and compared with those of the E2 cell fraction from cocultures of E2 and M3 (E2/M3). The collagenolytic activity in the culture medium of the E2 cells cultured alone was less than 8×10^{-3} units/ml, whereas that in the medium of the E2/M3 cocultures ranged from 40×10^{-3} to 100×10^{-3} units/ml. From Fig. *6A*, it can be seen that, other than gelatinase B mRNA, E2 cells cultured alone do not produce MMP mRNAs (collagenase-3, stromelysin-1, and stromelysin-2) at detectable levels. It can also be seen that this profile of gene expression does not change in the E2 cell fraction from E2/M3 cocultures. Thus, metaplastic cells were not required for the production of high levels of gelatinase B mRNA by E2 cells, although they may provide some degree of stimulation. Additionally, the mRNA levels of TIMP-1 and TIMP-2 did not differ between E2 cells and cocultured E2 cell fractions.

To determine whether the levels of MMP and TIMP mRNAs in metaplastic cells could be regulated by epithelial cells, the MMP and TIMP mRNA levels in M3 were measured and compared with those

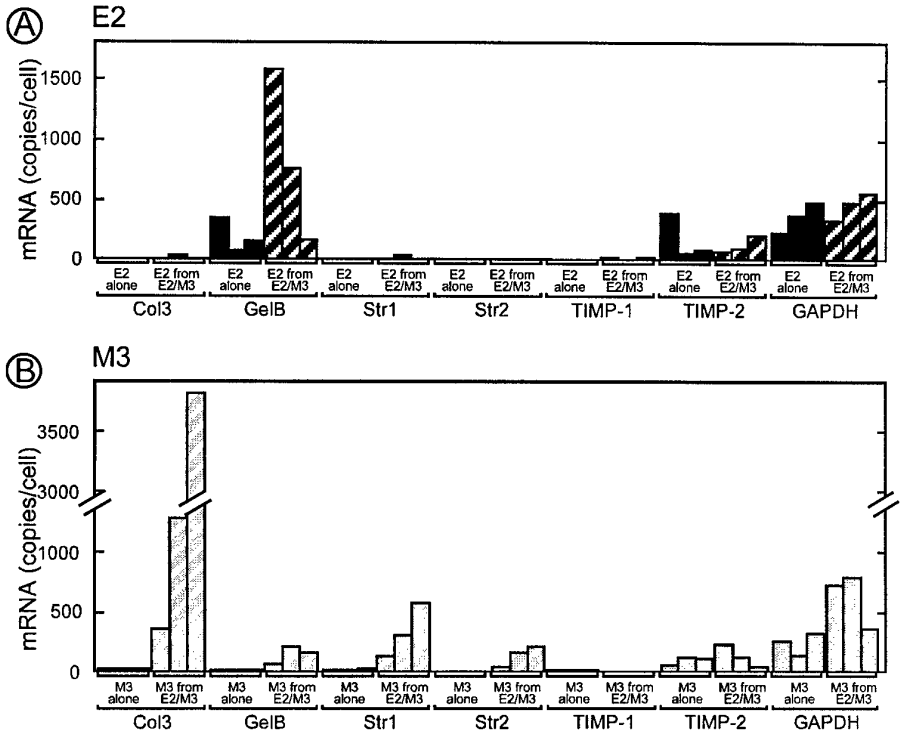

Fig. 6. Up-regulation of MMPs in metaplastic cells upon coculture with epithelial cells, mRNA levels for MMPs and TIMPs were determined by quantitative PCR analysis in E2 cells (*A*) and M3 cells (*B*) that had been grown in isolation (■) and in coculture (▨). The E2 and M3 cells from cocultures were isolated by differential trypsinization, as in Fig. 5. *Columns,* data from each of three individual experiments. *Col3,* collagenase-3; *GelB,* gelatinase B; *Str1,* stromelysin-1; *Str2,* stromelysin-2; *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase.

of the M3 cell fraction of E2/M3. M3 cells cultured in the absence of epithelial cells produced less than 8×10^{-3} units/ml collagenolytic activity in their medium. From this comparison (Fig. 6*B*), it can be seen that coculturing M3 with E2 effects a marked increase in the mRNA levels of collagenase-3, gelatinase B, stromelysin-1, and stromelysin-2 in M3 cells. Collagenase-3 mRNA levels in M3 cultures were never higher than 30 copies/cell, whereas the lowest value measured for collagenase-3 mRNA in the M3 cell fraction of E2/M3 cocultures was 350 copies/cell, and the highest value was 3800 copies/cell. Gelatinase B mRNA levels were elevated in the M3 cell fractions of E2/M3 cocultures, containing at least 65 copies/cell, when compared with the mRNA level in M3 cells cultured alone, which was never >7 copies/cell. Stromelysin-1 mRNA in M3 cells cultured alone was never >30 copies/cell, whereas in the M3 cell fraction of E2/M3 cocultures, the lowest and highest mRNA values measured were 128 and 570 copies/cell, respectively. Similarly, M3 cells cultured alone never produced more than two copies of stromelysin-2 mRNA/cell, whereas the M3 cell fractions of E2/M3 cocultures contained at least 40 copies/cell and as many as 200 copies/cell. TIMP-1 mRNA levels were slightly diminished upon coculture, were low in both cases, and TIMP-2 mRNA levels were high in both M3 cultured alone and the M3 cell fraction of E2/M3 cocultures.

DISCUSSION

We have shown that phenotypically distinct clones comprising mammary tumors can produce a high level of collagen degrading activity through interclonal induction of *MMP* gene expression. In BC1, cells that retained epithelial differentiation characteristics are required for induction of MMPs in cells from the same tumor that have undergone an epithelial-mesenchymal transition. Absolute quantitation of MMP and TIMP mRNAs has not been performed before, and has made it possible, for the first time, to compare accurately the mRNA levels of different MMPs and TIMPs within a cell or tissue sample and of the same mRNA between different samples. It has been determined by others that the mRNAs of <0.2% of genes expressed in mammalian cells are present at >500 copies/cell and that mRNAs of <2% are present at >50 copies/cell (29). Thus, the mRNAs of collagenase-3, gelatinase B, stromelysin-1, and TIMP-2 truly are major gene products of BC1 cells and tumors.

The genes for collagenase-3, gelatinase B, stromelysin-1, stromelysin-2, TIMP-1, and TIMP-2 were all expressed in the BC1 tumors, suggesting that each of them could have positive or negative roles in invasion and metastasis by BC1 *in vivo.* Previous reports in the literature regarding the likelihood that mammary carcinoma cells actively express these genes *in vivo* have varied and have only been qualitative in nature. The pattern of *MMP* and *TIMP* gene expression in BC1 cells *in vitro* was found to resemble closely that seen in BC1 tumors *in vivo* (Fig. 3). By histological analysis, it was determined that most cells in the tumors were neoplastic. Therefore, it is likely that most of the abundant mRNAs measured in the BC1 tumors were contributed by the BC1 cells themselves. Low level mRNAs (stromelysin-2 and TIMP-1) could be either abundant products of a low number of cells, such as macrophages and endothelial cells, or low level products of the BC1 cells. MMPs are produced as latent proenzymes that require activation by removal of specific propeptide sequences induced by exposure to other proteinases or chemicals (30). It has been demonstrated previously that BC1 cells secrete a plasminogen activator that initiates MMP activation and collagen degradation in the presence of plasminogen (31).

The *collagenase-3, stromelysin-1,* and *stromelysin-2* genes showed similar patterns of regulation in the mammary carcinoma cells in that metaplastic cells were the major source of these mRNAs and were induced by epithelial cells to up-regulate expression of the genes. The *collagenase-3* gene was expressed at a very high level in BC1 tumors *in vivo* and in cultures of BC1 *in vitro.* This is reflected in the large amounts of collagenase-3 protein produced by BC1 cells *in vitro* (32). The production of collagenase-3 by neoplastic cells of epithelial origin is consistent with the direct localization of collagenase-3 to

isolated mammary carcinoma cells by *in situ* hybridization, where the hybridization signal was found to be focal and intense (9). It is also consistent with the immunolocalization of collagenase-3 protein to the neoplastic cells of breast tumors (6), although another study by the same group identified stromal cells as the source of collagenase-3 by *in situ* hybridization of three tumor specimens (33).

The *stromelysin-1* gene was expressed at a high level in BC1 tumors *in vivo* and at a very high level in BC1 cells *in vitro*. Although some studies of stromelysin-1 in tumors have identified its origin as the stromal cells surrounding the tumor (9, 34, 35), others have observed the overexpression of the *stromelysin-1* gene by the neoplastic cells themselves, the stage of tumor progression being important in determining expression. In a study by Matrisian *et al.* (36), the *stromelysin-1* gene was shown to be expressed at higher levels in malignant than benign epidermal tumors. Further work investigating the transformation of squamous cell carcinomas to highly aggressive spindle cell carcinomas clearly demonstrated the expression of the *stromelysin-1* gene in the neoplastic cells of spindle cell carcinomas by *in situ* hybridization and immunolocalization, along with expression of the gene in the adjacent stroma (8). The squamous cell carcinomas, which represent the earlier stage of tumor progression in this model of multistage carcinogenesis, did not show expression of the gene within neoplastic cells, although stromal signals were abundant. Similarly, *in vitro* transformation of nonmetastatic squamous cell carcinoma cells by transfection results in spindle cells that are metastatic, express high levels of *stromelysin-1* gene, and cease expression of *keratin* genes (37, 38). Thus, in the epidermal carcinomas, *stromelysin-1* gene expression correlates directly with an epithelial-mesenchymal transition. Additionally, a direct association between stromelysin-1 mRNA production in mammary tissue and extracellular matrix remodeling has been suggested. An elevated level of stromelysin-1 mRNA was detected during involution of the mammary gland, a normal, physiological process that is well recognized as involving extensive matrix degradation (39). Myoepithelial cells, which represent the product of a normal developmental process involving a partial epithelial-mesenchymal transition, were identified as the source of stromelysin-1 in the involuting mammary gland, as well as in preneoplastic foci of *Ha-ras*-induced tumors (39).

Unlike the other MMPs, stromelysin-2 mRNA was present at low levels in BC1 tumors. This result is not surprising, considering the paucity of examples in the literature localizing expression of stromelysin-2 in neoplastic mammary tissue *in vivo*. However, stromelysin-2 has been identified previously in other tumors (40-42), and it appears to be selectively expressed in tissues of epithelial origin (43). Although it is not typically expressed at a high level in invasive neoplasms of the mammary gland (9), the detection of stromelysin-2 in BC1 tumors may be the result of the high level of sensitivity afforded by reverse transcription-PCR or by the stage of tumor progression.

BC1 tumors were found to contain substantial quantities of gelatinase B mRNA, although at levels that were consistently lower than collagenase-3 mRNA. In contrast to the other MMPs, epithelial cells were the main source of gelatinase B mRNA. Strong evidence for the involvement of gelatinase B in tumor metastasis is provided by a study where rat embryo cells, transformed with the *Ha-ras* and *E1A* genes and capable of forming nonmetastatic tumors, were converted to a metastatic phenotype by transient transfection with a gelatinase B expression plasmid (44). Several studies have implicated the involvement of gelatinase B in mammary carcinomas, specifically. It has been identified in human and rat breast cancer cell lines by reverse transcription-PCR or zymography (26, 45, 46) and has been immunolocalized to human breast carcinoma cells in tumor specimens (47-49). Although conflicting reports regarding the ability of mammary carcinoma cells to produce gelatinase B may be found in the literature (9, 49), the detection and quantitation of gelatinase B mRNA in BC1 cells and tumors provide further evidence that, at some stage of tumor progression, carcinoma cells can express the *gelatinase B* gene.

The level of TIMP-1 mRNA in BC1 cells and tumors was consistently low, whereas the level of TIMP-2 mRNA was very high. The absence of copurifying TIMP-1 in gelatinase B protein preparations isolated from E2 cultures on gelatin-Sepharose (26) is explained by the absence of TIMP-1 mRNA in E2 cells. The TIMP levels in BC1 are unlike those studies reporting either overexpression of the *TIMP-1* gene or similar levels of TIMP-1 and TIMP-2, where both have been investigated. TIMP-1 and TIMP-2 mRNA levels were shown to be elevated in a variety of tumor types including colorectal, pulmonary, and breast carcinoma (5, 39, 50) and were localized to both the stromal and tumor cells (9, 48). Down-regulation of *TIMP* genes can apparently confer a neoplastic phenotype upon immortalized cells (51). However, the invasiveness of BC1 cells coexists with a high level of *TIMP-2* gene expression. Clearly, then, production of TIMP-2 is not sufficient to prohibit metastasis. It may even contribute to extracellular matrix degradation by catalyzing the activation of MMPs at the cell surface (52, 53).

The behavior of tumors derived from the BC1 cell line was of interest with respect to its use as a model for investigating the expression of the genes of MMPs and TIMPs in the processes of invasion and metastasis, where they are believed to play important roles. The ability of tumors derived from BC1 cells to metastasize in an immunologically authentic host makes the model a particularly useful one. Previous studies have established the ability of BC1 tumors to invade bone, by means of inducing osteoclast-mediated resorption, and to metastasize to the draining lymph node (54, 55). In the present study, histological examination revealed their ability to invade the lumbricales muscles of the footpad and to metastasize spontaneously to the draining popliteal lymph node and to the lungs (Fig. 1). In these respects, the behavior of BC1 resembles that of human breast cancers, which usually metastasize via draining axillary lymph nodes to distant sites, such as bone, where invasion of tissues can occur. The presence of both epithelial and metaplastic cells within cell lines derived from lymph node metastases demonstrated that both cell types were capable of metastasizing. However, the absence of metaplastic cells from two of these cell lines is consistent with at least a subpopulation of BC1 epithelial cells being able to produce a lymph node metastasis independently of the presence of metaplastic cells in that lymph node.

Phenotypic heterogeneity is a common feature of neoplasms, despite their apparently clonal origin. It can arise through somatic changes in the genome (56, 57) or through normal differentiation processes (58-60). The BC1 cell line contains two readily discernible phenotypes. The epithelial cells have retained epithelial differentiation characteristics, including microvilli and tight junction formation (21), as well as expression of *E-cadherin* and *keratin* genes, whereas the metaplastic cells resemble an epithelial-mesenchymal transformation into the spindle cell or pseudosarcomatous component of carcinosarcoma-type breast carcinomas (15, 16, 28, 61). Metaplastic transformation of carcinoma cells, although well documented, is probably underdiagnosed, particularly in cases where the metaplastic cells have lost epithelial differentiation markers and do not form the majority of the tumor tissue. Loss of epithelial markers and acquisition of stromal markers in breast cancer are associated with hormone independence and a more aggressive behavior (17-20, 62), as well as a profile of *MMP* gene expression tending toward that of stromal cells (63, 64). In those cases, the neoplastic cells cannot readily be distinguished from tumor-associated stromal cells, particularly in human specimens, where it is not usually possible to isolate cells of stromal appearance

and test them for tumorigenicity, as was done for the metaplastic cells of BC1 (21). Ironically, the absence of epithelial markers and presence of stromal markers are often the same criteria used to identify MMP-producing cells in carcinomas as being stromal in origin. Thus, it is possible that some reports of *MMP* gene expression in carcinomas being confined to the nonneoplastic, tumor-associated stroma have actually detected expression in metaplastic carcinoma cells that have invaded the stroma. More definitive diagnostic tools for distinguishing between tumor-associated stromal and metaplastic cells in histological specimens would clarify this situation.

The present study shows for the first time that phenotypically distinct clones of cancer cells originating from the same tumor can cooperate through the modulation of gene expression of one cell type by the other, as exemplified by the up-regulation of the four *MMP* genes in metaplastic cells by epithelial cells. This ability of epithelial cells to induce the expression of the *MMP* genes in metaplastic cells suggests the existence of a signal originating with epithelial cells to which all four promoters are responsive in metaplastic cells. This could be either a soluble factor(s) or the direct result of cell-to-cell contact between metaplastic cells and epithelial cells. Transforming growth factor-α and emmprin are both examples of factors that have been associated with breast cancer cells and can induce production of MMPs in nonneoplastic cells (14, 65). Evidently, the genes of collagenase-3, stromelysin-1, and stromelysin-2 are not responsive to this signal in epithelial cells, where they are not expressed. Gelatinase B is unique among the MMPs examined in being produced at a constitutively high level by one of the cell types (the epithelial cells) in the absence of the other cell type. In contrast, the expression of its gene in metaplastic cells was dependent on induction by epithelial cells and was at a lower level, suggesting the possibility of two distinct modes of induction of this gene in cancer. Although other studies have shown that cancer cells can induce expression of MMP genes in nonneoplastic stromal cells *in vitro* (33, 34, 65), no previous study has demonstrated the ability of a subpopulation of neoplastic tumor cells to up-regulate expression of MMP genes in another subpopulation from the same tumor.

A second mechanism of interclonal cooperativity that was examined in BC1 is complementation of gene expression. In this case, the gene products of one or more clones do not exhibit an activity in isolation, but do so when mixed. This would have been evident by the culture media conditioned by epithelial cells and metaplastic cells that had been grown separately having no intrinsic collagenolytic activity, but acquiring it when mixed, because of a shift in balance between MMPs and TIMPs in favor of collagen degradation. However, this did not occur, demonstrating that this mechanism is insufficient to account for the acquisition of collagenolytic activity in mixed epithelial/metaplastic cell cultures. It remains a possibility that complementation of gene expression such as this may contribute to or even be necessary for interclonal cooperativity. However, it is insufficient in the absence of induction of MMP gene expression in metaplastic cells.

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Research Report

Absolute Quantitation of Specific mRNAs in Cell and Tissue Samples by Comparative PCR

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ABSTRACT

A comparative PCR assay, for the absolute quantitation of specific mRNAs in cell and tissue samples, has been designed to overcome problems with previous techniques. cDNAs made from the RNAs are co-amplified with "competitor" plasmid templates under conditions in which reagents are not limiting at the equivalence point, thereby preventing competition between target and competitor templates and distinguishing the assay from competitive PCR assays. The cDNAs are serially diluted, and competitor template concentrations are kept constant, rather than vice versa, as occurs in competitive PCR assays. Products from target and competitor templates are resolved by electrophoresis and measured by phosphorescent or fluorescent imagery. Both products are measured to minimize errors in the competitor:target ratio. A synthetic external standard RNA is included in the tissue lysis solution and co-purified with endogenous mRNAs, thereby being subject to identical losses of yield during subsequent procedures. The determination of the number of copies of external standard cDNA allows inefficiencies of RNA extraction and cDNA synthesis to be taken into account. Standard concentrations of plasmids containing the endogenous target sequences are also measured, so that corrections can be made for discrepancies due to unequal amplification of target and competitor sequences. These corrections, together with the use of an external standard and the PCR conditions chosen, allow for the accurate, specific and sensitive determination of the absolute number of mRNA copies in a sample.

INTRODUCTION

The amount of specific mRNAs within cells and tissues is important in the regulation of protein synthesis and is commonly used to study the expression of genes in various physiological and pathological processes. Several methods are commonly used to measure the levels of specific mRNAs, including northern blotting, primer extension, nuclease protection and reverse transcription-polymerase chain reaction (RT-PCR). As currently used, none of these methods permit absolute quantitation of mRNAs (i.e., they do not allow determination of the number of specific mRNA molecules in a particular cell culture or tissue sample). Relative levels of an mRNA are usually obtained by assaying the mRNA by one of the above-mentioned methods and normalizing the data against an internal standard RNA, usually ribosomal RNA or the mRNA of a "housekeeping" gene, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Even these relative levels rely on the internal standard RNA being expressed at a constant level in all tissues being examined. This can be an erroneous assumption, because even the expression of housekeeping genes are subject to cell-cycle and environmental regulation (5,9). Moreover, the validity of the relative levels relies on RNA extraction yields being constant between samples, an assumption that is rarely, if ever, verified. However, it would seem unlikely that the RNA yields from tissues with physical and biochemical differences would be identical.

In this paper, we describe a method based on competitive PCR (2,6,11,14) for the absolute quantitation of specific mRNAs that overcomes these problems. We call this method, "comparative PCR", as actual competition between the target and competitor templates does not occur. It uses non-limiting amplification conditions and a synthetic external standard RNA to take into account inefficiencies in RNA yield and cDNA synthesis. This method has been used to quantitate the mRNAs of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) in a mammary tumor.

MATERIALS AND METHODS

Synthetic External Standard RNA

To prepare the synthetic external standard RNA for quantitative PCR assays, the APL1 plasmid (described below) was linearized with *Eco*RI and transcribed with SP6 RNA Polymerase (Promega, Madison, WI, USA), according to the manufacturer's instructions. The RNA was electrophoresed in and eluted from 6.5% polyacrylamide/8 M urea gel and ethanol-precipitated. The purified RNA was quantitated by absorbance at 260 nm, re-analyzed electrophoretically to ensure integrity and stored under liquid nitrogen in aliquots until use.

Table 1. Absolute Quantitation of mRNAs in a Rat Mammary Tumor Sample

Gene		Primer Sequence		Product Size (bp)	Correction Factor	mRNA (millions of copies per mg tissue)
APL1	F	AGAAGTGTTTCAGAAGCTTCTCCC	T	344	1.02	-
	R	AACGAGCGGCTTCACTCAGACC	C	459		
Collagenase-3	F	CTCTCTATGGTCCAGG	T	144	1.44	210
	R	TCATGGTTTCTCCTCGG	C	159		
Gelatinase B	F	CGCCAACTATGACCAGGATA	T	73	0.976	167
	R	GTTGCCCCCAGTTACAGT	C	93		
Stromelysin-1	F	GCCTGGAATGGTCTTGG	T	222	1.28	96
	R	TGGAAACGGGCCAGGTC	C	195		
Stromelysin-2	F	GGAGTGGGACAGAGCTTGGC	T	312	1.59	33
	R	GACAGAGGGCACAGGAACCAC	C	225		
TIMP-1	F	AATTTGCACATCACTGCC	T	213	0.784	4
	R	GTGATCGCTCTGGTAGC	C	179		
TIMP-2	F	CAGGCGTTTTGCAATGC	T	114	1.32	16
	R	GATCTCATATTGAATCCTC	C	90		
GAPDH	F	CCACCATGGAGAAGGCTGGGGCTC	T	239	0.336	571
	R	AGTGATGGCATGGACTGTGGTCAT	C	278		

The sequences of the forward (F) and reverse (R) primers used for comparative PCR assays and the sizes of the products that they generate from cDNA target (T) and competitor (C) templates are shown. Comparative PCR assays were performed on three independent samples of known concentration of plasmid DNA or, for GAPDH, purified PCR product, containing the wild-type target sequences. The average discrepancy between the measured value and the actual value was determined as the correction factor. These values were used to correct the measurement of absolute numbers of mRNA copies in a BC1 tumor sample, determined by comparative PCR using the synthetic external standard RNA, as described in the text.

Extraction of RNA

Total RNA was extracted from cultured cells and tumors according to the method of Chomczynski and Sacchi (4), with the following modifications. External standard RNA, transcribed in vitro from the APL1 plasmid, was added to the guanidinium isothiocyanate (GITC) cell lysis solution before lysis at a concentration of 5.25×10^{-17} mol RNA per million cultured cells or per milligram of tissue. Tumors, kept frozen in dry ice, were pulverized using a mortar and pestle before the addition of cell lysis solution. The purity and quantity of total RNA extracted from samples was determined by spectrophotometric analysis at 260 and 280 nm.

Reverse Transcription

RT of mRNA was performed using 25 µg total RNA in a 100-µL reaction volume containing 100 U reverse transcriptase from the avian myeloblastosis virus (AMV) (Molecular Genetic Resources, Tampa, FL, USA), 1 mM deoxynucleotide triphosphates (Amersham Pharmacia Biotech, Castle Hill, NSW, Australia), 1.5 pg of 18-mer oligo(dT) and 100 U RNasin® Ribonuclease Inhibitor (Promega) in a buffer of 50 mM Tris-HCl, 8 mM $MgCl_2$, 30 mM KCl and 1 mM dithioerythritol, pH 8.5 (Boehringer Mannheim, Castle Hill, NSW, Australia). The reaction was carried out for 1 h at 42°C followed by 5 min at 98°C to inactivate the reverse transcriptase. The cDNA was subsequently stored at -70°C until assayed.

Comparative PCR

Table 1 shows the primer sequences for the quantitative PCR assays of each gene product. PCR was carried out in a 50-µL reaction volume containing 2.5 U of *Taq* DNA Polymerase (PE Biosystems, Scoresby, VIC, Australia), 0.2 mM deoxynucleotide triphosphates and 15 pmol each of forward and reverse primers in a buffer containing 10 mM Tris-HCl, 50 mM KCl, pH 8.35 (PE Biosystems). Reactions for comparative PCR in which phosphorescent imagery was used were supplemented with 0.6 µCi of [α-^{33}P]dCTP (Bresatec, Ade-

laide, SA, Australia) per reaction. When included, competitor plasmid was used at 0.1 amol per reaction. The standardized amplification protocol consisted of an initial denaturation step at 96°C for 4 min, followed by 25 sequential cycles of 96°C for 30 s, 54°C for 30 s and 72°C for 90 s. The optimal $MgCl_2$ concentration was determined for each primer pair to ensure specificity at the standard annealing temperature of 54°C. This was 0.8 mM for APL1, GAPDH and gelatinase B, 1.0 mM for stromelysin-2 and TIMP-1, 1.5 mM for collagenase-3 and TIMP-2 and 2.0 mM for stromelysin-1. Amplification was carried out in Omn-E or Omnigene® Thermal Cyclers (Hybaid, Ashford, England, UK). PCR products (10 µL) were electrophoresed in 12.5% or 15% polyacrylamide gels in a Mini-Protean® II Apparatus (Bio-Rad, Hercules, CA, USA). For phosphorescent imagery, the gels were dried, exposed to a phosphorescent screen overnight, imaged on a BAS1000 Phosphorescent Imager and quantitated with TINA software (both from Fuji, Tokyo, Japan). For fluorescent imagery, gels were stained for 30 min in SYBR® Green I (Molecular Probes, Eugene, OR, USA) or Vistra Green™ (Amersham Pharmacia Biotech), according to the manufacturers' instructions, imaged on a 312-nm UV light box with a charged-coupled device (CCD) camera and quantitated with Phoretix (both from UVP, Upland, CA, USA) or TINA software. In calculating the cDNA dilution at which target and competitor products were equivalent, differences in deoxycytosine content (for phosphorescent imaging) or total length (for fluorescent imaging) were taken into account. A molar factor of two was also included, to account for the fact that cDNA is single-stranded, whereas the competitor plasmids are double-stranded and therefore offer twice as many PCR templates.

Sequencing

Polyacrylamide gel-purified PCR products were sequenced directly using the Dye Terminator Cycle Sequencing Ready Reaction kit and a Model 373A Fluorescent Sequencer (both from PE Biosystems), according to the manufacturer's instructions.

Plasmids

APL1, the wild-type plasmid used for synthesis of the external standard RNA, was made by insertion of 539 bp of multiple synthetic oligonucleotides, including the sequences for PCR amplification of BCR-ABL, between the *Sal*I and *Sac*I sites of pSP64polyA (Promega). The competitor plasmids for this and other targets were made by insertion or deletion of DNA sequences between primer sites, using restriction enzymes or by deletions introduced by PCR primers (3) (Table 1). Complete sequences of the competitor plasmids are available upon request. Plasmids were purified by centrifugation through two cesium-chloride gradients.

Cell Culture and Tumors

The BC1 rat mammary carcinoma cell line was cultured under continuously serum-free conditions as described (8,12). Basal media and other culture chemicals were obtained from Sigma (St. Louis, MO, USA). Tumors were induced in 7-week-old syngeneic rats by injection of 0.5×10^6 cells in 50 µL of phosphate-buffered saline (PBS) into the right footpad and allowed to grow for 40 days.

RESULTS

Design of PCR Assays for Quantitating mRNAs

As part of an ongoing investigation into the regulation of extracellular matrix (ECM) destruction by tumors, it was necessary to quantitate MMP and TIMP mRNAs in the BC1 rat mammary carcinoma cell line and in tumors derived from it. To do this, a comparative PCR assay was designed that overcomes many of the problems associated with other mRNA assays. In particular, this protocol permits absolute quantitation of mRNA species, which eliminates the need to base measurements of the levels of the mRNA of interest on comparisons with those of other mRNAs, which may themselves be subject to modulation. In designing comparative PCR assays for the rat MMPs and TIMPs, the opportunity was taken to make methodological choices and innovations that would eliminate several deficiencies in existing quantitative

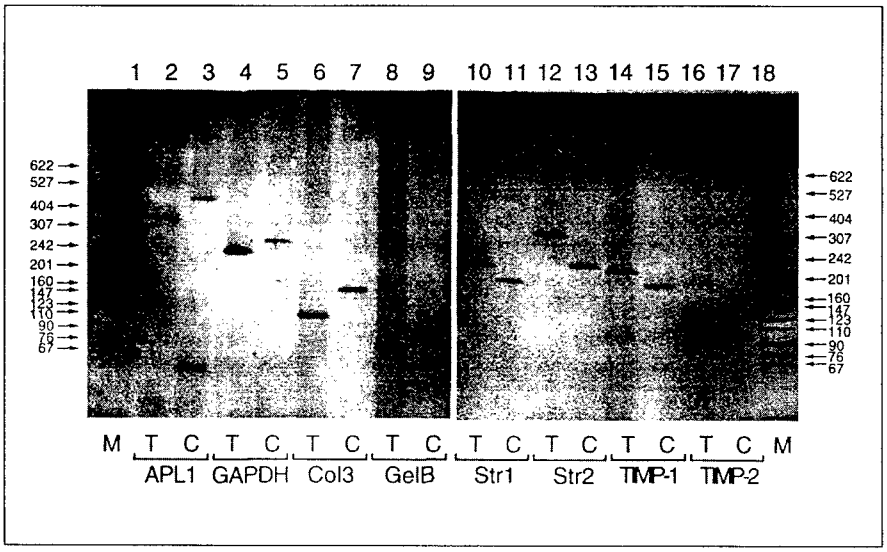

Figure 1. Specificity of PCRs for target and competitor templates. Silver-stained polyacrylamide gel electrophoretograms of target and competitor PCR products for each target mRNA, amplified under optimized conditions, are shown. All target products were amplified from BC1 cDNA except for the synthetic RNA standard, which was amplified from plasmid DNA. Competitor PCR products were amplified from the respective competitor plasmids. Lanes 2, 4, 6, 8, 10, 12, 14 and 16 show the specific target (T) products for the synthetic RNA standard (APL1), GAPDH, collagenase-3 (Col3), gelatinase B (GelB), stromelysin-1 (Str1), stromelysin-2 (Str2), TIMP-1 and TIMP-2, respectively. The corresponding competitor PCR products (C) for each are shown in the adjacent lanes (i.e., lanes 3, 5, 7, 9, 11, 13, 15 and 17, respectively). A primer dimer band is evident in lane 3. Lanes 1 and 18 are size markers, with the number of base pairs indicated beside the gels.

PCR methods. Four of these were of particular importance: (*i*) the introduction of a known amount of APL1, a synthetic external standard RNA at the beginning of RNA extraction. The quantitation of APL1 cDNA in samples permitted inefficiencies of RNA recovery and cDNA synthesis in the samples to be calculated and taken into account; (*ii*) to titrate the cDNA samples against a fixed amount of competitor, rather than vice versa. This ensured that the total amount of template DNA for a given primer set was constant for all of

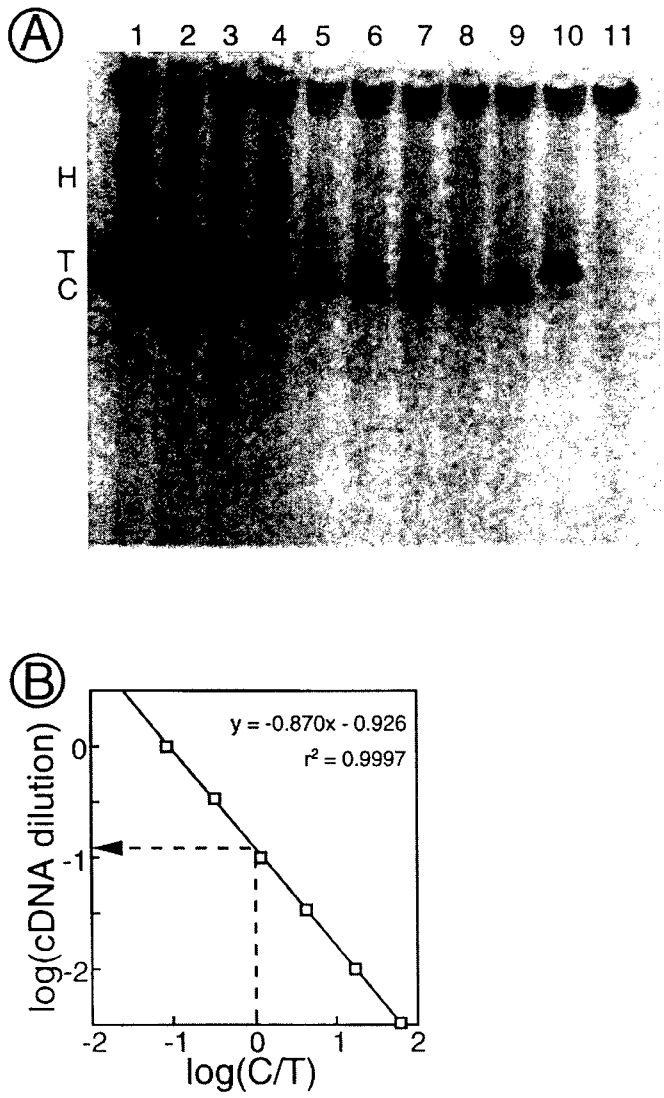

Figure 2. Comparative PCR analysis of cDNA. (A) Phosphorescent image of a polyacrylamide gel showing the PCR products in a comparative RT-PCR assay for TIMP-1. Dilutions of sample cDNA were co-amplified by PCR with 0.1 amol of competitor, in the presence of [^{33}P]dCTP. PCR products were separated by 15% polyacrylamide gel electrophoresis (PAGE) and visualized by phosphorescent imagery. Competitor (C), target (T) and heteroduplex (H) PCR products are indicated. Lanes 1, 3, 5 and 7 show the products arising from 10-fold serial dilutions of the cDNA. For each 10-fold dilution, an additional 3-fold dilution was performed, the products arising from these being shown in lanes 2, 4, 6 and 8, respectively. (B) Graphical determination of the amount of TIMP-1 cDNA. The intensities of the bands in Panel A were quantitated and corrected for background intensity. The logarithm of the dilution of target was plotted against the logarithm of the ratio of competitor value to target value for each dilution (lanes 1–6). The equation describing the line of best fit, obtained by the least squares method, and the regression coefficient of the line are presented.

the samples at the dilution at which equivalent amounts of target and competitor products were generated; (*iii*) to choose PCR conditions such that reactions at the point of equivalence were maintained in the exponential phase of amplification throughout the reaction; and (*iv*) to use a cDNA of known concentration to determine the degree to which amplification bias of target over competitor or vice versa affected the determination of the point of equivalence. The result was a sensitive method that allowed the determination of absolute numbers of copies of MMP and TIMP mRNAs in cell cultures and tissue samples. For the sake of convenience, standard thermal cycling parameters were chosen, so that assays for several mRNAs could be performed simultaneously in a single block.

Comparative PCR assays were developed for the quantitation of the mRNAs encoding rat collagenase-3, gelatinase B, stromelysin-1, stromelysin-2, TIMP-1, TIMP-2, GAPDH and the synthetic external standard, APL1. Figure 1 shows the corresponding target and competitor-derived PCR products for each of the MMPs and TIMPs investigated. The specificity of each primer pair was evaluated empirically on BC1 rat mammary carcinoma-derived cDNA and competitor templates. Under the optimized PCR conditions utilized, the primer pairs for each MMP and TIMP specifically amplified products of the expected size. Occasionally, bands corresponding to primer dimer formation were visible. However, these did not interfere with quantitation, as assays were performed under conditions in which reagents were not limiting (see Discussion). In addition to correct size, product identities were verified by direct sequencing. All of the MMP and TIMP sequences were identical to those that have been published or submitted to sequence databanks, except for stromelysin-1. GenBank® Accession No. X02601 reads C-C at bases 623 and 624, whereas BC1-derived stromelysin-1 cDNA reads A-A. This alters the predicted amino acid at codon 189 from threonine in the original sequence to isoleucine.

Figure 2A shows a phosphorescent image of a comparative PCR assay, in which the bands corresponding to target (T) and competitor (C) products were generated. In this case, the target was the low-abundance mRNA for TIMP-1. Note that, as the amount of cDNA added to the reaction increased, the intensity of the bands of the competitor product remained constant, until the amount of cDNA increased past the point of equivalence, indicating that reagents were not limiting at the point of equivalence. Figure 2A, lanes 9 and 10, show the competitor-only and cDNA-only controls, respectively, demonstrating the specificity of the reaction and absence of contamination.

In addition to the expected target and competitor products, a third band or doublet of DNA (labeled H in Figure 2A, lanes 1–4) was routinely seen in comparative PCR assays for each MMP and TIMP, in reactions at or near the equivalence point. Although the identity of the third band(s) was unknown, its appearance was not the result of a non-specific priming event, as it consistently failed to appear in both the competitor-only and the cDNA-only PCR controls (Figure 2A, lanes 9 and 10). It was hypothesized that this third band might be the result of the formation of heteroduplexes between target and competitor products, because its occurrence was maximal near the equivalence point. To verify this, the DNA constituting the additional band was analyzed in two ways. First, gel-purified material from both homoduplex bands and the putative heteroduplex band (Figure 3A, lanes 2–4) were denatured by heating to 96°C and then allowed to renature by cooling slowly to room temperature. Under these conditions, a heteroduplex will separate at the higher temperature into single strands. Upon cooling, each target sense strand can re-anneal with antisense strands from either target or competitor products, thereby forming both homoduplexes and heteroduplexes. Similarly, the target antisense strand and both strands of the competitor product will form both homoduplexes and heteroduplexes, upon cooling. Figure 3A, lane 5, shows that bands corresponding in mobility to both heteroduplexes and homoduplexes were formed upon renaturing the putative heteroduplex product, confirming its identity as a heteroduplex. Control samples of target and competitor homoduplexes reannealed to give bands with their original mobilities (Figure 3A, lanes 6 and 7). Secondly, gel-purified homoduplex and putative heteroduplex DNAs were electrophoresed under denaturing conditions. A heteroduplex will dissociate into its constituent target and competitor single strands when denatured. Figure 3B shows that the putative heteroduplex dissociates into two clusters of bands (lane 2), with mobilities corresponding to those resulting from the dissociation of the target and competitor products into single strands (Figure 3B, lanes 3 and 4).

Phosphorescent imagery and fluorescent imagery were both suitable for the quantitation of the PCR products. Phosphorescent imagery had the advan-

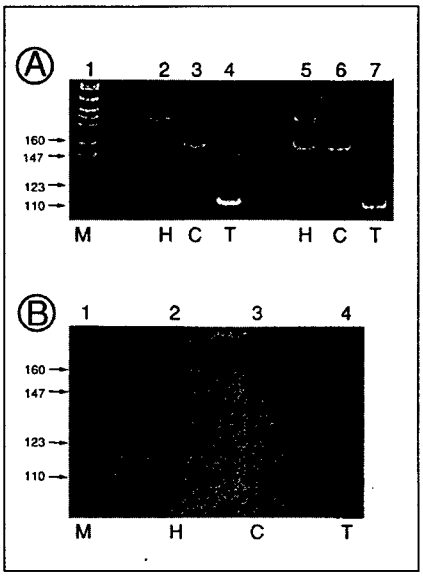

Figure 3. Heteroduplex formation in comparative PCRs. (A) Non-denaturing PAGE of renatured PCR products. Heteroduplex (H), target (T) and competitor (C) collagenase-3 PCR products were gel-purified. Each product was denatured by heating to 96°C and then cooled slowly for renaturation. The products before denaturation (lanes 2–4) and following renaturation (lanes 5, 6 and 7) were visualized by staining with ethidium bromide. The generation of target and competitor bands from the heteroduplex band is evident in lane 5. Lane 1 contains size markers, with the number of base pairs indicated to the left of the gel. (B) Gel-purified target, competitor and heteroduplex PCR products were electrophoresed in a denaturing polyacrylamide gel. Whereas the target (lane 4) and competitor (lane 3) products resolved into their respective complementary strands, the heteroduplex (lane 2) was revealed to be composed of both target and competitor strands. Lane 1 contains size markers, with the number of base pairs indicated to the left of the gel.

tage of its response to signal intensity being linear over several orders of magnitude. The regression coefficients of the lines of best fit of data obtained by phosphorescent imagery were always greater than 0.9, and the slopes were usually between -0.85 and -1.2 (Figure 2B). A slope of -1 is predicted for this type of PCR assay (10). The small deviations from -1 might be due to variations in the measurement of background signal, because a small adjustment of the background level, by adding or subtracting a fixed amount of signal from all values, caused a substantial change in the slope, while causing just a very small change in the Y-intercept value.

The use of fluorescent imagery required that care be taken that the image be captured within the linear response range of the CCD camera, which was limited to approximately three orders of magnitude. Under these conditions, the lines of best fit were similar to those obtained by phosphorescent imagery. To ensure that measurements were taken consistently within the linear response range of the CCD camera, the signal strength of the competitor band in the competitor-only lane in each assay was adjusted by changing the aperture setting of the camera to give a fixed signal output corresponding to the middle of the linear response range. Fluorescent imagery had the advantage over phosphorescent imagery of speed, in not having to dry the gels and expose them overnight to an imaging screen, and avoided the expense and hazard of radioactive isotopes. Both imaging methods had equivalent sensitivity under the conditions used.

In comparative PCR, a discrepancy between the measured value and the actual value for an mRNA species might result when there is a difference in amplification efficiency between target and competitor templates (10). Therefore, the accuracy of each comparative PCR assay was determined by performing comparative PCR with known amounts of purified competitor and target templates, so that the discrepancies between actual and measured values could be accounted for in assays of samples. The discrepancy, or mean fold error, between the measured value and expected value for each target template, was 1–3-fold for the 8 assays (Table 1). These values were subsequently used to correct the target measurements made on cDNA samples. In this way, the values were adjusted for the amplification bias intrinsic to each assay and also for the incorporation of products into heteroduplexes.

To verify that the mRNA quantitation was independent of the amount of starting material, TIMP-2 mRNA was quantitated in the BC1-E1 clonal cell line (8), starting with 2.5×10^6, 25×10^6 and 50×10^6 cells. Six samples of each cell number were independently reverse transcribed and amplified and produced values of 102 ± 40, 122 ± 28 and 137 ± 39 copies/cell, respectively. Thus, the outcome of the assay for a given mRNA and cell type did not differ significantly, regardless of the amount of starting material over at least a 20-fold range, suggesting that any variations in the yield of RNA due to different amounts of starting material had been taken into account by the use of the external standard.

Expression of MMP and TIMP Genes in Mammary Tumors In Vivo

The comparative PCR assay incorporating the external standard was used to determine the absolute quantities of MMP and TIMP mRNAs in a sample of a rat mammary tumor (Table 1). High levels of mRNAs encoding the MMPs were detected, ranging from $33–210 \times 10^6$ copies per milligram tissue. Because each milligram of tissue contains approximately 10^6 cells, as determined by the density of packed cultured cells, this corresponds to approximately 33–210 copies per cell. By comparison, the TIMP mRNAs were present at lower levels, and the biologically unrelated GAPDH mRNA was present at 571×10^6 copies per milligram tissue. It has been estimated that less than 2% of expressed genes are expressed at greater than 50 mRNA copies per cell (13). Thus, collagenase-3, stromelysin-1 and gelatinase B can be said to be expressed at high levels in this tumor.

DISCUSSION

We have described a protocol for the quantitation of mRNAs in cell and tissue samples that overcomes deficien-

cies in previous methods, which either do not offer absolute quantitation or do not take into account losses of yield during RNA isolation and cDNA synthesis. The current generally acceptable practice for examining RNA levels is to generate a specific signal using a technique (usually northern blotting) and compare it with the corresponding signal from the product of a housekeeping gene, such as GAPDH. This does not allow absolute quantitation of the mRNA of interest and, even for comparisons of relative mRNA levels to be made, assumes a uniform level of expression of the housekeeping gene in the different tissues or treatments of cells being compared. It is perhaps surprising, given its long history of use as a standard by which the expression of other genes are compared, that the gene for GAPDH was isolated only in 1988 (5) and that, in that paper, its expression was demonstrated to be modulated by insulin. Thus, cells that differ in their responsiveness to insulin or that grow in different concentrations of insulin or similar effectors would produce different amounts of GAPDH mRNA, thereby invalidating its use as a gene of invariant expression. Earlier work had demonstrated tissue-specific variations in the levels of GAPDH mRNA (9). It is likely that other commonly used internal standard genes undergo regulation that is dependent on environmental influences, tissue specificity or cell cycle.

Absolute quantitation of mRNA levels using the current protocol was made possible by the design of an assay that incorporated the following set of attributes: (*i*) the introduction of a synthetic external standard RNA; (*ii*) the titration of target against a constant amount of competitor, rather than vice versa; (*iii*) the use of PCR conditions in which reagents were not limiting and (*iv*) and the verification of the accuracy of the competitor assays, using known amounts of targets. No previous assay has incorporated all of these attributes, which are essential to the accurate quantitation of mRNAs in cell and tissue samples.

The inclusion of the external standard allowed inefficiencies and inconsistencies of RNA extraction and RT to be taken into account, a feature that is essential to absolute quantitation, but which has not been addressed by previous mRNA assays of cell and tissue samples, although it has been used in the semiquantitation of in vitro transcripts (1). Without the external standard, it is not possible to take into account losses of mRNA during purification and incomplete cDNA synthesis, thereby making absolute quantitation impossible. If variability of mRNA yields occurs between tissues, even relative quantitation is impossible without knowing the extent to which

the variability has occurred. The introduction of the external standard RNA to account for these errors assumes an identical behavior of the external standard and the mRNA being measured. Thus, it is important to ensure that the cell or tissue, from which the RNA is being extracted, is lysed completely and homogeneously in the GITC solution, and that reverse transcriptase, primers and nucleotides are not limiting during cDNA synthesis.

Most published protocols for competitive PCR assays determine the point of equivalence by co-amplifying a constant amount of sample against a titration of competitor (6). This is an aspect of PCR assays that has not received much attention previously, but is worthy of scrutiny because it has important consequences for the accuracy of the assay. During PCR, reagents become limiting when cycling continues after a certain amount of product has been amplified. When this occurs, the post-exponential phase of PCR is reached, and the bias in amplification efficiency between the target and competitor sequences, brought about by differences in length and sequence composition, becomes more pronounced, so that the amplification of one template is favored over the other (10). This amplification bias introduces a discrepancy between the actual and measured amounts of target that increases with cycle number. Those PCRs having a higher amount of total template DNA (target + competitor) will reach plateau phase earlier than those having a lower amount of total template DNA. Thus, the amplification bias and resultant discrepancies in measurements will be different for PCRs containing different amounts of total template DNA (10). Therefore, in PCR assays in which a constant amount of target-containing sample is co-amplified against a titration of competitor, the amount of total template DNA amplified at the point of equivalence will be different for each sample, resulting in different discrepancies between measured and actual values for each sample. Thus, in those assays, different correction factors would be needed for each different concentration of sample.

The above situation can be avoided by co-amplifying a constant amount of competitor against a titration series of sample, as is done in the present protocol. Using this protocol, the amount of total template DNA amplified at the point of equivalence is equal for all samples, regardless of their target cDNA content. Consequently, the discrepancy between actual and measured amounts of target, due to amplification bias, is constant for all samples and can be taken into account, once it has been determined empirically. Also, by restricting the PCR to 25 cycles, the reagents do not become limiting for the amount of competitor chosen (0.1 amol) (Figure 2). As a result, the bias in amplification efficiency between target and competitor sequences is minimized (10), and small amounts of nonspecific products do not interfere, because actual competition for reagents does not occur. To this extent, competitive PCR is a misnomer for assays conducted within the exponential phase of amplification. Even greater sensitivity can often be achieved by reducing the amount of competitor to 0.01 amol and increasing the cycle number to 30.

Although the use of gel electrophoresis to distinguish the products of the two templates is somewhat labor-intensive, it has the advantage of being able to measure the products of both the target and the competitor templates in each sample. Under conditions in which reagents are not limiting, such as those used in the present protocol, it is necessary to measure both products. PCR assays that detect only one product measure the product of the template that is added in a fixed amount and rely on competition between it and the template being titrated to determine the point of equivalence. Thus, those types of assays must be taken beyond the exponential phase of amplification, since competition requires that reagents become limiting, and consequently, those types of assays must be subject to the potential problems associated with entry to the post-exponential phase, such as increasing amplification biases. By separating the products electrophoretically and measuring them both to determine their ratio, the necessity for entry into plateau phase is avoided. Another advantage of measuring both products is that it diminishes the so-called "tube effect", in which otherwise identical PCRs can generate different amounts of

products in different tubes, due to the nonuniformity of heating blocks and tube shapes. Because the ratio of both products is being measured, and the change in amplification efficiency due to the tube effect is likely to affect both templates equally during the exponential phase, the ratio will not be affected. In contrast, if just one product is being measured, errors due to the tube effect will be incorporated into the data.

Verification of the equivalence point is important in establishing the accuracy of any competitive or comparative PCR assay, but is rarely reported. The amplification bias for each pair of target and competitor templates in the assays described here were determined empirically from a comparative PCR assay on a solution containing a known concentration of purified DNA incorporating the target sequence. The known concentration was compared with the measured concentration, and the proportional error gave a measure of the amplification bias over 25 cycles. That the discrepancy was always less than threefold confirmed that the bias for one template over another was small (<5% per cycle) under the conditions chosen. Importantly, irrespective of its size, this error factor can be taken into account in subsequent assays and is essential to ensuring accuracy of quantitation.

Absolute quantitation is important to understanding gene expression, as it will enable comparisons of mRNA levels to be made between different mRNAs within a sample, between different types of cells and tissues, between different time points and between different laboratories. Also, it enables transcription initiation rates to be determined (7). The use of the comparative PCR procedure described here avoids problems associated with previously described methods and also contributes a greater sensitivity and specificity than non-PCR-based techniques.

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